

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

03

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification⁵ : C12N 15/29, C12P 21/08 A61K 39/36	A1	(11) International Publication Number: WO 93/04174 (43) International Publication Date: 4 March 1993 (04.03.93)
(21) International Application Number: PCT/AU92/00430 (22) International Filing Date: 14 August 1992 (14.08.92) (30) Priority data: 746,702 16 August 1991 (16.08.91) US (71) Applicant: THE UNIVERSITY OF MELBOURNE [AU/ AU]; Grattan Street, Parkville, VIC 3052 (AU). (72) Inventors: SINGH, Mohan, Bir ; 7 Lloyd Court, Temples- towe, VIC 3106 (AU). HOUGH, Terryn ; 25 Bowman Street, Mordialloc, VIC 3195 (AU). KNOX, Robert, Bruce ; 9 Riverview Road, North Balwyn, VIC 3104 (AU). THEERAKULPISUT, Piyada ; 1/74 Canning Street, Carlton, VIC 3053 (AU). SMITH, Penelope ; 80 Best Street, North Fitzroy, VIC 3052 (AU). AVJIOGLU, Asil ; 23 Rathmullen Quadrant, Doncaster, VIC 3108 (AU). ONG, Eng, Kok ; 2/1 Moffat Street, South Yarra, VIC 3141 (AU).		(74) Agents: SLATTERY, John, M. et al.; Davies & Collison, 1 Little Collins Street, Melbourne, VIC 3000 (AU). (81) Designated States: AU, CA, DK, JP, KR, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, SE). Published <i>With international search report.</i>
(54) Title: RYEGRASS POLLEN ALLERGEN (57) Abstract The present invention provides nucleic acid sequences coding for two ryegrass pollen allergen <i>Lol p</i> Ib family members, purified <i>Lol p</i> Ib.1 and <i>Lol p</i> Ib.2 proteins and fragments thereof, methods of producing recombinant <i>Lol p</i> Ib.1 or <i>Lol p</i> Ib.2 or at least one fragment thereof or derivative or homologue thereof, and methods of using the nucleic acid sequences, proteins and peptides of the invention.		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FI	Finland	MN	Mongolia
AU	Australia	FR	France	MR	Mauritania
BB	Barbados	GA	Gabon	MW	Malawi
BE	Belgium	GB	United Kingdom	NL	Netherlands
BF	Burkina Faso	GN	Guinea	NO	Norway
BG	Bulgaria	GR	Greece	NZ	New Zealand
BJ	Benin	HU	Hungary	PL	Poland
BR	Brazil	IE	Ireland	PT	Portugal
CA	Canada	IT	Italy	RO	Romania
CF	Central African Republic	JP	Japan	RU	Russian Federation
CG	Congo	KP	Democratic People's Republic of Korea	SD	Sudan
CH	Switzerland	KR	Republic of Korea	SE	Sweden
CI	Côte d'Ivoire	LI	Liechtenstein	SK	Slovak Republic
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CS	Czechoslovakia	LU	Luxembourg	SU	Soviet Union
CZ	Czech Republic	MC	Monaco	TD	Chad
DE	Germany	MG	Madagascar	TG	Togo
DK	Denmark	ML	Mali	UA	Ukraine
ES	Spain			US	United States of America

- 1 -

RYEGRASS POLLEN ALLERGEN**FIELD OF THE INVENTION**

The present invention relates to allergenic proteins from pollen of ryegrass, *Lolium perenne* L., and fragments, derivatives and homologues thereof, and to allergenic proteins immunologically related thereto. More particularly, the present invention relates to the major allergenic protein family Lol p Ib from pollen of ryegrass and related proteins of the Lol p Ib protein family.

10 REFERENCE TO RELATED APPLICATIONS

The present application is a continuation-in-part application of U.S. application Serial no. 746,702, filed August 16, 1991 which is, in turn, a continuation-in-part application of U.S. application Serial no. 585,086 filed March 24, 1990, the disclosures of both of which are hereby incorporated by reference.

BACKGROUND OF THE INVENTION

Genetically predisposed individuals, who make up about 10% of the population, become hypersensitized (allergic) to antigens from a variety of environmental sources to which they are exposed. Those antigens that can induce immediate and/or delayed types of hypersensitivity are known as allergens. (King, T.P., Adv. Immunol. 23 77-105 (1976) Anaphylaxis or atopy, which includes the symptoms of hay fever, asthma, and

SUBSTITUTE SHEET

- 2 -

hives, is one form of immediate allergy. It can be caused by a variety of atopic allergens, such as products of grasses, trees, weeds, animal dander, insects, food, drugs and chemicals.

5 The antibodies involved in atopic allergy belong primarily to the IgE class of immunoglobulins. IgE bonds to mast cells and basophils. Upon combination of a specific allergen with IgE bound to mast cells or basophils, the IgE may be cross-linked on the cell surface, resulting in the
10 physiological effects of IgE-antigen interaction. These physiological effects include the release of, among other substances, histamine, serotonin, heparin, a chemotactic factor for eosinophilic leukocytes and/or the leukotrienes, C4, D4 and E4, which cause prolonged constriction of bronchial smooth
15 muscle cells (Hood, L.E. et al. *Immunology*, 2nd ed.) The Benjamin/Cumming Publishing Co., Inc., (1984). These released substances are the mediators which result in allergic symptoms caused by a combination of IgE with a specific allergen. Through them, the effects of an allergen are manifested. Such
20 effects may be systematic or local in nature, depending on the route by which the antigen entered the body and the pattern of deposition of IgE on mast cells or basophils. Local manifestations generally occur in epithelial surfaces at the location at which the allergen entered the body. Systemic
25 effects can include anaphylaxis (anaphylactic shock), which is the result of an IgE-basophil response to circulating (intravascular) antigen.

 Allergens constitute the most abundant proteins of grass pollen, which is the major cause of allergic disease in
30 temperate climates (Marsh (1975) Allergens and the genetics of allergy; in M. Sela (ed), *The Antigens*, Vol. 3, pp 271-359, Academic Press Inc., London, New York)., Hill et al. (1979) *Medical Journal of Australia* 1, 426-429). The first descriptions of the allergenic proteins in ryegrass showed that
35 they are immunochemically distinct, and are known as groups I, II, III and IV (Johnson and March (1965) *Nature*, 206, 935- ; and Johnson and Marsh (1966) *Immunochemistry* 3, 91-100). Using

SUBSTITUTE SHEET

- 3 -

the International Union of Immunological Societies' (IUIS) nomenclature, these allergens are designated *Lol p Ib*, *Lol p II*, *Lol p III* and *Lol p IV*. However, the allergenic spectrum of ryegrass pollen is now known to be more complex. The
5 international reference preparation for ryegrass contains 17 allergens ranging in molecular weight from 12 to 89kD (Stewart et al. (1988) Int. Arch. Allergy Appl. Immunol. 86: 9-18). These allergenic proteins in pollen have been detected by their ability to bind IgE, the immunoglobulin specifically present in
10 allergic individuals.

Among these allergens, *Lol p I*, *II*, *III* and *IV* have been extensively studied. The full amino acid sequences of *Lol pII* and *III* have been reported. This is made possible by using standard biochemical techniques due to the high amount of
15 allergenic proteins in the pollen and the relatively small molecular weight of the proteins. Although the proteins of *Lol p I* and *IV* are abundant in the pollen, only partial amino acid sequences had been reported using the same techniques. This is due to the relatively high molecular weight of the proteins.
20 In addition, it is difficult to purify allergens without any cross-contamination and is labor-intensive. Lack of primary sequence and highly purified allergens in sufficient quantity have been the limiting factor in the development of both therapeutic and diagnostic products for the treatment and
25 diagnosis of type I allergies.

Lol p I is defined as an allergen because of its ability to bind to specific IgE in sera of ryegrass-sensitive patients, to act as an antigen in IgG responses and to trigger T-cell responses. The allergenic properties have been assessed
30 by direct skin testing of grass pollen-sensitive patients. The results showed that 84% had a skin-sensitivity to *Lol pI* (Freidhoff et al., (1986) J. Allergy Clin. Immunol. 78: 1190-1201), demonstrating the primary importance of this protein as the major allergen. Furthermore, 95% of patients demonstrated
35 to be grass pollen-sensitive possessed specific IgE antibody that bound to *Lol pI*, as demonstrated by immunoblotting (Ford and Baldo (1986) International Archives of Allergy and Applied

SUBSTITUTE SHEET

- 4 -

Immunology 81: 193-203).

Substantial allergenic cross-reactivity between grass pollens has been demonstrated using an IgE-binding assay, the radioallergo-sorbent test (RAST), for example, as described by
5 Marsh et al. (1970) J. Allergy, 46, 107-121, and Lowenstein (1978) Prog. Allergy, 25, 1-62. (Karger, Basel).

The immunochemical relationship of *Lol p I* with other grass pollen antigens have been demonstrated using both polyclonal and monoclonal antibodies (e.g. Smart and Knox
10 (1979) International Archives of Allergy and Applied Immunology 62: 173-187; Singh and Knox (1985) International Archives of Allergy and Applied Immunology 78, 300-304). Antibodies have been prepared to both purified proteins and IgE-binding components. These data demonstrate that the major allergen
15 present in pollen of closely related grasses is immunochemically similar to *Lol p I* (Singh and Knox, *supra*).

SUMMARY OF THE INVENTION

In accordance with the present invention, it has been discovered that the ryegrass pollen allergen *Lol p I* comprises
20 two proteins, designated as herein *Lol p Ia* and *Lol p Ib*. The *Lol p Ib* ryegrass pollen allergen is present in *L. perenne* as a family of proteins. The genes encoding two family members of *Lol p Ib*, designated *Lol p Ib.1* and *Lol p Ib.2* have now been identified. Family member *Lol p Ib.1* was previously designated
25 *Lol p Ib* and is now referred to as *Lol p Ib.1*. As used herein, *Lol p Ib* thus refers to a major ryegrass pollen protein allergen which is actually a family of closely related proteins having similar structure and function but encoded by separate genes. Therefore, the terms *Lol p Ib* and *Lol p Ib* family
30 members may be used herein interchangeably.

The present invention provides purified nucleic acid sequences coding for at least one *Lol p Ib* ryegrass pollen allergen, or at least one antigenic fragment thereof, or derivative or homologue thereof, or the functional equivalent
35 of said nucleic acid sequence. The present invention also provides expression vectors comprising a nucleic acid sequence

SUBSTITUTE SHEET

- 5 -

coding for at least one *Lol p Ib* ryegrass pollen allergen, or at least one antigenic fragment thereof, or derivative or homologue thereof, or the functional equivalent of said nucleic acid sequence. The present invention further provides host
5 cells transformed to express a protein or peptide encoded by the nucleic acid sequences of the invention.

Another aspect of the present invention provides at least one purified *Lol p Ib* ryegrass pollen allergen, or at least one antigenic fragment thereof, or derivative or
10 homologue. A further aspect of the present invention provides an isolated antigenic fragment of an allergen from ryegrass pollen, preferably from a *Lol p Ib* ryegrass pollen allergen. More preferably the ryegrass pollen allergen is *Lol p Ib.1* or *Lol p Ib.2*.

15 Still another aspect of the invention provides a modified ryegrass pollen protein allergen which, when administered to a ryegrass pollen-sensitive individual, reduces the allergic response of the individual to ryegrass pollen. Preferably the ryegrass pollen allergen is a modified *Lol p Ib*
20 protein or derivative or homologue thereof. More preferably the ryegrass pollen allergen is a modified *Lol p Ib.1* or *Lol p Ib.2* protein or derivative or homologue thereof. The present invention also provides at least one modified fragment of ryegrass pollen protein allergen which, when administered to a
25 ryegrass pollen-sensitive individual, reduces the allergic response of the individual to ryegrass pollen. Preferably the ryegrass pollen allergen is a *Lol p Ib* ryegrass pollen allergen, more preferably *Lol p Ib.1* or *Lol p Ib.2*. Isolated protein allergen or antigenic fragment thereof immunologically
30 related to *Lol p Ib.1* or *Lol p Ib.2*, or fragment or derivative or homologue of *Lol p Ib.1* or *Lol p Ib.2* is also provided by the present invention.

In yet another aspect of the present invention, there is provided non-native (i.e., recombinant or chemically
35 synthesized) *Lol p Ib* family members or their derivatives or homologues, or a non-native allergenic protein immunologically cross-reactive to antibodies to one or more *Lol p Ib* family

SUBSTITUTE SHEET

- 6 -

members or their derivatives or homologues. The present invention also provides purified native Lol p Ib proteins or at least one fragment or derivative or homologue thereof.

Non-native Lol p Ib protein, and fragments or portions derived therefrom (peptides) can be used in methods of diagnosing, treating and preventing allergic reactions to ryegrass pollen. Purified native Lol p Ib protein, fragments thereof, and homologues or derivatives thereof are also useful in methods of diagnosing, treating and preventing allergic reactions to ryegrass pollen.

Still yet another aspect of the present invention relates to antibodies to non-native Lol p Ib or derivatives or homologues thereof as well as antibodies raised against purified native Lol p Ib or derivatives or homologues thereof.

Further features of the present invention will be better understood from the following detailed description of the preferred embodiments of the invention in conjunction with the appended figures.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows isolation of cDNA clones specific for the Poaceae Group Ib allergens. Figure 1a illustrates recognition of a positive clone (12R) by three different MABs FMC A1 (40.1), FMC A7 (12.3), 3.2 (Kahn & Marsh (1986) *Molec. Immunol.* 23: 1281-1288; Singh & Knox (1985) *International Archives of Allergy and Applied Immunology* 78, 300-304; Smart et al. (1983) *International Archives of Allergy and Applied Immunology* 72 243-248) and IgE from allergic patients' sera. C is the control in which the primary MAb was omitted. Figure 1b shows an immunoblot analysis of MABs and IgE binding to group I antigens from rye-grass pollen. Lane 1 shows total protein profile (Coomassie blue staining); Lane 2: MAB 21.3; Lane 3: MAB 40.1; Lane 4: MAB 3.2; Lane 5 12.3-; Lane 6: IgE antibodies.

Figure 2 shows tissue-type and cell-type specific expression of group Ib allergen transcripts. Figure 2a shows RNA blot hybridization. Poly(A)+ RNAs were isolated from

SUBSTITUTE SHEET

- 7 -

different plant tissues: seed leaf, root and pollen. Figure 2b shows immunoblot analysis of tissue-type and cell-type specific distribution of group Ib antigens. The soluble proteins were extracted from different plant tissues: flower, leaf root and pollen, and were immunoblotted using MAbs 40.1 (panel 1), 12.3 (panel 2) and IgE antibodies (panel 3).

Figure 3 shows the cDNA sequence, predicted amino acid sequence and hydrophilicity profile of rye-grass pollen clone 12R, designated *Lol p Ib.1*. Figure 3a shows a schematic restriction map of lambda-12R cDNA. The hatched box represents the predicted translation open reading frame. Figures 3b and 3c show the nucleotide and deduced amino acid sequence of the 1229 nucleotide EcoRI cDNA insert lambda-12R. The deduced amino acid sequence represented by the single letter code is shown below the DNA sequence, and begins at the first potential in-frame initiation codon at nucleotide 40. One uninterrupted open reading frame continues for 301 amino acids (numbered below the DNA sequence) and ends with the TGA stop codon denoted by an asterisk. The putative signal peptide is indicated by negative numbers. The amino acid residues 1-9, 12-17, and 19 were identified by N-terminal sequencing. Figure 3d shows the hydrophilicity profile of predicted amino acid sequence based on method of Hopp and Woods (1981) *Proc. Natl Acad. Sci. USA* 78: 3824-3828, with a window of seven amino acids.

Figure 4 shows the delineation of IgE and MAb-reacting epitopes in *Lol p Ib.1* (clone 12R) using immunoblotting: Figure 4a: IgE antibodies; Figure 4b, MAb 40.1 and Figure 4c, MAb 12.3. Controls for Figures 4a-c are provided by bacteria transformed with non-recombinant plasmids.

Figure 5 shows detection of *Lol p Ia* and *Lol p Ib* in mature pollen of rye-grass using specific MAbs and immunogold probes. Figure 5a shows whole pollen grains visualized by scanning electron microscopy, showing the single germinal pore. Scale bar, 30 um. Figure 5b shows detection of cellular sites of *Lol p Ia* and *Lol p Ib* by immuno-gold localization - double labelling. Figure 5c shows the appearance of fresh, viable

SUBSTITUTE SHEET

- 8 -

pollen after exposure to water for 30s, dark field illumination.

Figure 6 shows antibody binding to non-denatured pollen proteins from 20 different grasses. Lanes A through E shown binding of various antibodies to the pollen proteins in the listed grass species - lane A) IgE, lane B) FMC-A1 antibody, lane C) FMC-A-7 antibody, lane D) LpIX-3A antibody, and lane E) LpIX-4A antibody.

Figure 7 shows soluble pollen proteins of 20 different grasses separated by gradient SDS-PAGE and visualized by Coomassie Brilliant Blue R250 staining (Figure 7a), and binding of serum IgE (Figure 7b), monoclonal antibody FMC-A1 (Figure 7c), monoclonal antibody FMC-A7 (Figure 7d), antibody LpIX-3A (Figure 7e) and antibody LpIX-4A (Figure 7f). Individual lanes correspond to the grasses shown in Figure 6.

Figure 8 is a photographic representation of immunoscreening of clone 19R in the vector lambda gt 11 using pooled sera from (a) allergic patients and (b) non-allergic patients.

Figure 9 is a schematic representation showing a partial restriction endonuclease map of *Lol p Ib.2* (clone 19R), *Lol p Ib.1* (clone 12R) and *Lol p Ia*.

Figures 10a and 10b show the cDNA sequence and predicted amino acid sequence of *Lol p Ib.2* (clone 19R).

Figure 11 is a graphical representation of a hydrophobicity profile of the predicted amino acid sequence for *Lol p Ib.2* based on the method of Kyte and Doolittle (1982) *J. Mol. Biol.*, 157:105-132, with a window of nine amino acids.

Figures 12a and 12 are a representation showing comparison of the cDNA sequences of *Lol p Ib.2* (clone 19R) and *Lol p Ib.1* (clone 12R). A bar is used to show identity between DNA sequences. Gaps are inserted within the translated region to show maximum similarity. The number of gaps inserted in clone 19R is 14. The number of gaps inserted in clone 12R is 35. Overall sequence identity is 887 bases (72.2%).

Figure 13 is a representation comparing amino acid sequences of *Lol p Ib.2* (clone 19R) and *Lol p Ib.1* (clone 12R).

SUBSTITUTE SHEET

- 9 -

Gaps are inserted within the translated region to show maximum similarity. A bar is used to show identity between amino acid sequences and "s" shows similarity between amino acid sequences. Amino acids said to be "similar" are A, S and T; D and E; N and Q; R and K; I, L, M and V; and F, Y and W. The number of gaps inserted in clone 19R is 1. The number of gaps inserted in clone 12R is 4. The two sequences contain 201 identical amino acids (66.8%), and 38 "similar" amino acids (12.6%).

10 Figure 14 shows tissue-specific expression of clone 19R. Figure 14(a) Northern blot analysis of total RNA from ryegrass pollen, leaf, root and seed probed with a 82 base fragment specific to clone 19R. Figure 14(b) Northern dot blot analysis of total RNA from ryegrass pollen, leaf, root and seed
15 probed with a ribosomal DNA from *Pisum sativum*. 20 ug of total RNA was loaded in Figures 14(a) and (b) for all tissues.

Figure 15 represents pollen proteins probed with sera of allergic individuals in a two-dimensional Western blot analysis. Group Ia is components 1-4; Group Ib is components
20 5-12; Group IV is components 13-15; Group II is components 16-17 and Group III is ?.

Figure 16 shows two-dimensional Western analysis of ryegrass pollen proteins. In all cases, ryegrass pollen proteins were subjected to isoelectric focussing (left to
25 right) followed by SDS-PAGE (top to bottom). (a) two-dimensional gel electrophoresis separation of total proteins silver stained. Two-dimensional Western blots probed with (b) total IgE antibodies from pooled sera of grass pollen allergic patients, (c) MAb FMC-A7, (d) IgE antibodies
30 affinity-purified from Lol p Ib.1 and (e) IgE antibodies affinity-purified from Lol p Ib.2.

Figure 17 shows dot blot screening of lambda gt 11 Lol p Ib.1 (clone 12R), Lol p Ia (clone 13R) and Lol p Ib.2 (clone 19R) on E. coli Y1090. Two microliters of phage stocks of
35 clones 12R, 13R and 19R and a non-recombinant lambda gt 11 were spotted onto a lawn of E. coli Y1090 induced with a nitrocellulose filter saturated with 10 mM IPTG. The protein

SUBSTITUTE SHEET

- 10 -

blots were then probed with individual serum from 30 grass allergic patients. a=Lol p Ib.1, b=Lol p Ia, c=Lol p Ib.2 and d= non-recombinant lambda gt 11. [<] = individuals having higher level of IgE bound to Lol p Ib.2 than Lol p Ia and Lol p Ib.1. [1] = individuals having IgE specific to Lol p Ia. [2] = individuals having IgE specific to Lol p Ib.1 and Lol p Ib.2.

DETAILED DESCRIPTION OF THE INVENTION

The data herein show that what was considered to be the major allergen of rye-grass pollen, Lol p I, actually comprises at least two different allergenic proteins: Lol p Ia, which comprises 4 different isoforms in the 35 kD range with pIs ranging from about 5.5-7.0 and Lol p Ib, which comprises at least 5 different isoforms of 31/33 kD proteins, and pIs ranging from 6.0-10.6. Lol p Ib has a different primary structure and composition from Lol p Ia, as deduced from NH₂-terminal amino acid sequence and the absence of allergenic cross-reactivity. cDNA clones encoding Lol p Ib.1 (clone 12R) and Lol p Ib.2 (clone 19R) have been isolated and characterized. The Lol p Ib protein encoded by clones 12R and 19R have a different primary structure and composition from Lol p Ia, as deduced by cDNA cloning and the absence of allergenic cross-reactivity. The NH₂-terminal sequence of recombinant Lol p Ib.1 is identical to that determined for purified native Lol p Ib. However, Lol p Ib.1 and Lol p Ib.2 are apparently acidic proteins, having a predicted pI of 5.16 and 5.9, respectively. Purified native Lol p Ib, Lol p Ib.1 and Lol p Ib.2 are non-glycosylated proteins with similar molecular weights (31/33 kD) and similar NH₂ terminal sequences. These similarities suggest that the genes encoding the native Lol p Ib and recombinant Lol p Ib proteins will be different members of the same gene family. Lol p Ib family members are synthesized in pollen as a preallergen with a 25 amino acid signal peptide that targets the allergen to plastids. This is followed by cleavage of the peptide, and in mature pollen the allergen occurs predominantly in the starch grains.

Thus, one aspect of the present invention provides

SUBSTITUTE SHEET

- 11 -

purified nucleic acid sequences coding for at least one *Lol p Ib* ryegrass pollen allergen, or at least one antigenic fragment thereof, or derivative or homologue thereof, or the functional equivalent of such nucleic acid sequence. Preferred nucleic acid sequences coding for *Lol p Ib* family members include the nucleic acid sequence encoding amino acids -25 through 276 of *Lol p Ib.1* as shown in Figures 3b and 3c, and the nucleic acid sequence encoding amino acids -25 through 314 of *Lol p Ib.2* shown in Figures 10a and 10b. These sequences encode the entire *Lol p Ib.1* protein and *Lol p Ib.2* protein, including the 25 amino acid signal peptide. Other preferred nucleic acid sequences include the nucleic acid sequence encoding amino acids 1-276 of *Lol p Ib.1* shown in Figures 3b and 3c, and the nucleic acid sequence encoding amino acids 1-314 of *Lol p Ib.2* shown in Figures 10a and 10b. These nucleic acid sequences encode the mature *Lol p Ib.1* and *Lol p Ib.2* proteins. Still other nucleic acid sequences of the invention include nucleic acid sequences encoding at least one fragment of the coding portion of the nucleic acid sequence of *Lol p Ib.1* shown in Fig. 3b-3c, or at least one fragment of the coding portion of the nucleic acid sequence of *Lol p Ib.2* shown in Figures 10a and 10b, or the functional equivalent of such nucleic acid sequences.

The original source of the genetic material is fresh ryegrass pollen from *Lolium perenne* L., collected from field sources near Melbourne, Australia and bulk collected pollen from a supplier (Greer Laboratories, Lenoir, NC) and from flowerhead. These sources of pollen are not intended to limit the scope of the invention since they only represent one convenient supply of the pollen. The present invention can be practiced using pollen from any location.

"Gene", is used, in respect of the present invention, in its broadest sense and refers to any contiguous sequence of nucleotides, the transcription of which leads to a mRNA molecule, which mRNA molecule is capable of being translated into a protein. The gene encoding a *Lol p Ib* family member means the nucleotide sequence encoding the protein or

SUBSTITUTE SHEET

- 12 -

derivatives or homologues of the protein which may contain single or multiple amino acid substitutions, deletions or additions. A *Lol p Ib* gene also refers to cDNAs complementary to the mRNAs corresponding to the full or partial length of a
5 *Lol p Ib* protein.

It is expected that there are sequence polymorphisms in the nucleic acid sequence coding for each *Lol p Ib* family member, and it will be appreciated by one skilled in the art that one or more nucleotides in the nucleic acid sequence
10 coding for a *Lol p Ib* family member may vary among individual *L. perenne* plants due to natural allelic variation. Any and all such nucleotide variations and resulting amino acid polymorphisms are within the scope of the invention. It may also be appreciated by one skilled in the art that *Lol p Ib* is
15 a family of highly related genes whose proteins are present in *L. perenne* pollen (e.g. Rafnar et al. (1991) *J. Biol. Chem.* 266: 1229-1236; Silvanovich et al. (1991) *J. Biol. Chem.* 266: 1204-1210). Nucleotide sequences and corresponding deduced amino acid sequences of any and all such related family members
20 including *Lol p Ib.1* and *Lol p Ib.2* are within the scope of the invention.

Accordingly, it is within the scope of the present invention to encompass all proteins belonging to the *Lol p Ib* family, at least one fragment (peptide) of a *Lol p Ib* protein
25 family member, and amino acid derivatives thereof, and to encompass nucleotide sequences, including DNA, cDNA and mRNA and homologue or degenerate forms thereof, encoding *Lol p Ib* family members or fragments thereof, or derivatives thereof. It is also within the scope of the invention to encompass
30 purified native *Lol p Ib*, at least one fragment (peptide) thereof, and derivatives or homologues thereof. It is further in accordance with the present invention to include molecules such as polypeptides fused to a *Lol p Ib* protein, or at least one fragment thereof, or derivatives thereof or to nucleotide
35 sequences contiguous to such fragment and/or derivative-encoding nucleotide sequences. For example, for some aspects of the present invention, it is desirable to

SUBSTITUTE SHEET

- 13 -

produce a fusion protein comprising a *Lol p Ib* family member or at least one fragment thereof or their derivatives and an amino acid sequence from another peptide or protein, examples of the latter being enzymes such as beta-galactosidase, phosphatase, urease and the like. Most fusion proteins are formed by the expression of a recombinant gene in which two coding sequences have been joined together such that their reading frames are in phase. Alternatively, proteins or peptides can be linked *in vitro* by chemical means. All such fusion protein or hybrid genetic derivatives of a *Lol p Ib* protein or its encoding nucleotide sequences are encompassed by the present invention. Furthermore, by homologues and derivatives of a *Lol p Ib* protein is meant to include synthetic derivatives thereof. The nucleotide sequences as elucidated herein, can be used to chemically synthesize the entire protein or generate any number of fragments (peptides) by chemical synthesis by well known methods (eg solid phase synthesis). All such chemically synthesized peptides are encompassed by the present invention. Accordingly, the present invention extends to isolated *Lol p Ib* protein family members, fragments thereof and their derivatives, homologues and immunological relatives made by recombinant means or by chemical synthesis.

The terms "isolated" and "purified" are used interchangeably herein and refer to peptides, protein, protein fragments, and nucleic acid sequences substantially free of cellular material or culture medium when produced by recombinant DNA techniques, or chemical precursors or other chemicals when synthesized chemically. The term "native purified" as used herein refers to proteins or fragments thereof purified from *L. perenne* pollen or other plant part. Furthermore, the present invention extends to proteins or fragments (peptides) corresponding in whole or part to the nucleotide coding sequence given in Figures 3b and 3c and Figures 10a and 10b, or to degenerate or homologue forms thereof.

Fragments of nucleic acid within the scope of the invention include those coding for parts of *Lol p Ib* that

SUBSTITUTE SHEET

- 14 -

elicit an immune response in mammals, preferably humans, such as the stimulation of minimal amounts of IgE; binding of IgE; eliciting the production of IgG and IgM antibodies; or the eliciting of a T cell response such as proliferation and/or lymphokine secretion and/or the induction of T cell anergy. The foregoing fragments of *Lol p Ib* are referred to herein as antigenic fragments. Fragments within the scope of the invention also include those capable of hybridizing with nucleic acid from other plant species for use in screening protocols to detect allergens that are cross-reactive with *Lol p Ib* protein. As used herein, a fragment of the nucleic acid sequence coding for *Lol p Ib* refers to a nucleotide sequence having fewer bases than the nucleotide sequence coding for the entire amino acid sequence of *Lol p Ib* and/or a mature *Lol p Ib* family member. Generally, the nucleic acid sequence coding for the fragment or fragments of a *Lol p Ib* family member will be selected from the bases coding for the mature *Lol p Ib* protein family member, however, in some instances it may be desirable to select all or a part of a fragment or fragments from the leader sequence portion of a nucleic acid sequence of the invention. A nucleic acid sequence of the invention may also contain linker sequences, restriction endonuclease sites and other sequences useful for cloning, expression or purification of a *Lol p Ib* protein or fragments thereof.

Antigenic fragments of an allergen from ryegrass pollen, preferably *Lol p Ib.1*, *Lol p Ib.2*, or purified native *Lol p Ib*, may be obtained, for example, by screening peptides produced by recombinant methods from the corresponding fragment of the nucleic acid sequence of the invention coding for such peptides, synthesized chemically using techniques known in the art, or by degrading of the purified allergen. The peptide fragments of the protein allergen may be obtained by any method known in the art such as chemical cleavage of the allergen, arbitrary division of the allergen into fragments of a desired length with no overlap of the peptides, or preferably division of the allergen into overlapping fragments of a desired length. The fragments are tested to determine their antigenicity and

- 15 -

allergenicity. Fragments of recombinantly or synthetically produced Lol p Ib or of purified native Lol p Ib which are capable of eliciting a T cell response such as stimulation (i.e., proliferation or lymphokine secretion) and/or are capable of inducing T cell anergy are particularly desirable. Fragments of recombinantly or synthetically produced Lol p Ib or purified native Lol p Ib which do not bind immunoglobulin E (IgE) and/or which have minimal IgE stimulating activity are also desirable. If the fragment or fragments of a recombinantly or synthetically produced Lol p Ib protein family member or purified native Lol p Ib bind IgE, it is preferable that such binding does not lead to histamine release, e.g., such binding does not cause cross-linking of IgE on mast cells or basophils. Minimal IgE stimulating activity refers to IgE stimulating activity that is less than the amount of IgE production stimulated by whole recombinantly or synthetically produced Lol p Ib protein or whole purified native Lol p Ib protein. Preferred fragments also include antigenic fragments which, when administered to a ryegrass pollen-sensitive individual or an individual allergic to an allergen cross-reactive with ryegrass pollen allergen, are capable of modifying the allergic response to ryegrass pollen allergen of the individual, and antigenic fragments which, when administered to a ryegrass pollen-sensitive individual, are capable of modifying B-cell response, T-cell response or both B-cell and T-cell response of the individual to a ryegrass pollen allergen. As used herein modification of the allergic response of an individual sensitive to ryegrass pollen allergen can be defined as non-responsiveness or diminution in symptoms to the allergen, as determined by standard clinical procedures (see e.g. Varney et al, *British Medical Journal*, (1990), 302:265-269), including diminution in grass pollen induced asthmatic symptoms (Suphioglu et al. (1992) *Lancet* 339: 569-572).

Antigenic fragments of the present invention which have T cell stimulating activity, and thus comprise at least one T cell epitope are particularly desirable. T cell epitopes are believed to be involved in initiation and perpetuation of

SUBSTITUTE SHEET

- 16 -

the immune response to a protein allergen which is responsible for the clinical symptoms of allergy. These T cell epitopes are thought to trigger early events at the level of the T helper cell by binding to an appropriate HLA molecule on the surface of an antigen presenting cell and stimulating the relevant T cell subpopulation. These events lead to T cell proliferation, lymphokine secretion, local inflammatory reactions, recruitment of additional immune cells to the site, and activation of the B cell cascade leading to production of antibodies. One isotype of these antibodies, IgE, is fundamentally important to the development of allergic symptoms and its production is influenced early in the cascade of events, at the level of the T helper cell, by the nature of the lymphokines secreted. A T cell epitope is the basic element or smallest unit of recognition by a T cell receptor, where the epitope comprises amino acids essential to receptor recognition. Amino acid sequences which mimic those of the T cell epitopes and which modify the allergic response to protein allergens are within the scope of this invention.

Exposure of patients to purified protein allergens of the present invention or to the antigenic fragments of the present invention which comprise at least one T cell epitope and are derived from protein allergens may tolerize or anergize appropriate T cell subpopulations such that they become unresponsive to the protein allergen and do not participate in stimulating an immune response upon such exposure. In addition, administration of the protein allergen of the invention or an antigenic fragment of the present invention which comprises at least one T cell epitope may modify the lymphokine secretion profile as compared with exposure to the naturally-occurring protein allergen or portion thereof (e.g. result in a decrease of IL-4 and/or an increase in IL-2). Furthermore, exposure to such antigenic fragment or protein allergen may influence T cell subpopulations which normally participate in the response to the allergen such that these T cells are drawn away from the site(s) of normal exposure to the allergen (e.g., nasal mucosa, skin, and lung) towards the

SUBSTITUTE SHEET

- 17 -

site(s) of therapeutic administration of the fragment or protein allergen. This redistribution of T cell subpopulations may ameliorate or reduce the ability of an individual's immune system to stimulate the usual immune response at the site of
5 normal exposure to the allergen, resulting in a diminution in allergic symptoms.

Screening for IgE binding to the protein or fragments thereof may be performed by scratch tests or intradermal skin tests on laboratory animals or human volunteers, or in *in vitro*
10 systems such as RAST (radioallergosorbent test), RAST inhibition, ELISA assay or radioimmunoassay (RIA).

The present invention provides expression vectors and host cells transformed to express the nucleic acid sequences of the invention. Expression vectors of the invention comprise a
15 nucleic acid sequence coding for at least one *Lol p Ib* ryegrass pollen allergen, or at least one antigenic fragment thereof, or derivative or homologue thereof, or the functional equivalent of such nucleic acid sequence. Nucleic acid sequences coding for *Lol p Ib* family members including *Lol p Ib.1* or *Lol p Ib.2*,
20 or at least one fragment thereof may be expressed in prokaryotic or eukaryotic host cells. Suitable host cells include bacterial cells such as *E. coli*, insect cells, yeast, or mammalian cells such as Chinese hamster ovary cells (CHO). Suitable expression vectors, promoters, enhancers, and other
25 expression control elements may be found in Sambrook et al. *Molecular Cloning: A Laboratory Manual*, second edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1989. Suitable vectors for expression in yeast include *YepSec1* (Baldari et al. (1987) *Embo J.* 6: 229-234); *PMF₋* (Kurjan and
30 Herskowitz (1982) *Cell* 30: 933-943); and *JRY88* (Schultz et al. (1987) *Gene* 54: 113-123).

For expression in *E. coli*, suitable expression vectors include *pTRC* (Amann et al. (1988) *Gene* 69: 301- 315); *pET-11d* (Novagen, Madison, WI); *pGEX* (Amrad Corp., Melbourne,
35 Australia); *pMAL* (N.E. Biolabs, Beverly, MA); *pRIT5* (Pharmacia, Piscataway, NJ); and *pSEM* (Knapp et al. (1990) *BioTechniques* 8: 280-281). The use of *pTRC* and *pET-11d* will lead to the

SUBSTITUTE SHEET

- 18 -

expression of unfused protein. The use of pGEX, pMAL, pRIT5 and pSEM will lead to the expression of allergen fused to glutathione S-transferase (pGEX), maltose E binding protein (pMAL), protein A (pRIT5), or truncated β -galactosidase (PSEM).

5 When a *Lol p Ib* protein family member, fragment, or fragments thereof is expressed as a fusion protein, it is particularly advantageous to introduce an enzymatic cleavage site at the fusion junction between the carrier protein and the *Lol p Ib* protein family member or fragment thereof. A *Lol p Ib* family member or fragment thereof may then be recovered from the fusion protein through enzymatic cleavage at the enzymatic site and biochemical purification using conventional techniques for purification of proteins and peptides. Suitable enzymatic cleavage sites include those for blood clotting Factor Xa or thrombin for which the appropriate enzymes and protocols for cleavage are commercially available from for example Sigma Chemical Company, St. Louis, MO and N.E. Biolabs, Beverly, MA.

10
15

Host cells can be transformed to express the nucleic acid sequences of the invention using conventional techniques such as calcium phosphate or calcium chloride co-precipitation, DEAE-dextran-mediated transfection, or electroporation. Suitable methods for transforming the host cells may be found in Sambrook et al. *supra*, and other laboratory textbooks. The nucleic acid sequences of the invention may also be synthesized using standard techniques.

20
25

Accordingly, another aspect of the present invention provides a method of producing recombinant *Lol p Ib.1* or *Lol p Ib.2*, or at least one fragment thereof, or their derivatives or homologues, or their immunological relatives (as hereinbefore defined) comprising culturing an organism containing a replicable recombinant DNA molecule, said molecule comprising a promoter capable of expression in said organism, a gene encoding a *Lol p Ib* family member, at least one fragment thereof, or homologue or derivative thereof, or immunological relatives thereof, located downstream of and transcribed from said promoter, a selectable marker and a DNA vehicle containing a prokaryotic or eukaryotic origin of replication, under

30
35

SUBSTITUTE SHEET

- 19 -

conditions and for a time sufficient for said recombinant DNA molecule to be stably maintained and direct the synthesis of the Lol p Ib protein, at least one fragment thereof, or derivatives, homologues or immunological relatives thereof and
5 then optionally isolating same.

Lol p Ib.1 protein, Lol p Ib.2 protein and fragments (peptides) thereof can be purified from cell culture medium, host cells, or both using techniques known in the art for purifying peptides and proteins, including ion-exchange
10 chromatography, gel filtration chromatography, ultrafiltration, electrophoresis and immunopurification with antibodies specific for Lol p Ib.1, Lol p Ib.2 or fragment of Lol p Ib.1 or Lol p Ib.2. The terms isolated and purified are used interchangeably herein and refer to peptides, protein, protein fragments, and
15 nucleic acid sequences substantially free of cellular material or culture medium when produced by recombinant DNA techniques, or chemical precursors or other chemicals when synthesized chemically.

Another aspect of the invention provides protein
20 preparations comprising Lol p Ib, Lol p Ib.1, Lol p Ib.2 or at least one fragment of Lol p Ib, Lol p Ib.1 or Lol p Ib.2. In preferred embodiments of this aspect of the invention, Lol p Ib.1 or Lol p Ib.2 protein or at least one fragment of Lol p Ib.1 or Lol p Ib.2 is produced in a host cell transformed with
25 a nucleic acid sequence coding for the protein or fragment.

Using the structural information now available, it is possible to design peptides derived from Lol p Ib which, when administered to a ryegrass pollen sensitive individual in sufficient quantities, will modify the individual's allergic
30 response to ryegrass pollen. This can be done, for example, by examining the structure of Lol p Ib, producing peptides (via an expression system, synthetically or otherwise) to be examined for their ability to influence B-cell and/or T-cell responses in ryegrass pollen sensitive individuals and selecting
35 appropriate epitopes recognized by the cells. In referring to an epitope, the epitope will be the basic element or smallest unit of recognition by a receptor, particularly

SUBSTITUTE SHEET

- 20 -

immunoglobulins, histocompatibility antigens and T cell receptors where the amino acids essential to the receptor recognition may be contiguous and/or non-contiguous in the amino acid sequence. Amino acid sequences which mimic those of the epitopes and which are capable of down regulating allergic response to ryegrass pollen allergen can also be used.

It is now also possible to design an agent or a drug capable of blocking or inhibiting the ability of ryegrass pollen allergen to induce an allergic reaction in ryegrass pollen sensitive individuals. Such agents could be designed, for example, in such a manner that they would bind to relevant anti-Lol p Ib-IgE's, thus preventing IgE-allergen binding and subsequent mast cell or basophil degranulation. Alternatively, such agents could bind to cellular components of the immune system, resulting in suppression or desensitization of the allergic response to *L. perenne* pollen allergens. A non-restrictive example of this is the use of appropriate B- and T-cell epitope peptides, or modifications thereof, based on the cDNA/protein structures of the present invention to suppress the allergic response to ryegrass pollen. This can be carried out by defining the structures of B- and T-cell epitope peptides which affect B- and T-cell function in *in vitro* studies with blood components from ryegrass pollen sensitive individuals.

Protein, peptides or antibodies of the present invention can also be used for detecting and diagnosing ryegrass pollinosis. For example, this could be done by combining blood or blood products obtained from an individual to be assessed for sensitivity to ryegrass pollen with an isolated antigenic peptide or peptides of recombinantly or synthetically produced Lol p Ib or native purified Lol p Ib, or isolated Lol p Ib protein or isolated native purified Lol p Ib protein, under conditions appropriate for binding of components (e.g., antibodies, T-cells, B-cells) in the blood with the peptide(s) or protein and determining the extent to which such binding occurs. The extent to which binding occurs can be determined, for example, by assessing T cell function, T cell

SUBSTITUTE SHEET

- 21 -

proliferation, B cell function, or binding of the protein, or fragment thereof, or derivative or homologue thereof to antibodies present in the blood or a combination thereof.

Additionally, sensitivity of a mammal to ryegrass pollen may be determined by administering to a mammal a sufficient quantity of at least one ryegrass pollen allergen belonging to the *Lol p Ib* family, or at least one antigenic fragment thereof, or derivative or homologue thereof to provoke an allergic response in the mammal and determining the occurrence of an allergic response in the mammal to the ryegrass pollen allergen. The ryegrass pollen allergen(s), fragment(s) or derivative or homologue thereof used in this aspect of the present invention can be produced recombinantly or synthetically. Purified native *Lol p Ib* protein or fragments thereof may be substituted for a recombinantly or synthetically produced *Lol p Ib* or fragments thereof and used in the above method to determine sensitivity of the mammal to ryegrass.

The DNA used in any embodiment of this invention can be cDNA obtained as described herein, or alternatively, can be any oligodeoxynucleotide sequence having all or a portion of a sequence represented herein, or their functional equivalents. Such oligodeoxynucleotide sequences can be produced chemically or mechanically, using known techniques. A functional equivalent of an oligonucleotide sequence is one which is 1) a sequence capable of hybridizing to a complementary oligonucleotide to which the sequence (or corresponding sequence portions) of Figures 3b and 3c (*Lol p Ib.1*) or fragments thereof hybridizes or to which the sequence (or corresponding sequence portions) of Figures 10a and 10b (*Lol p Ib.2*) or fragments thereof hybridizes, or 2) the sequence (or corresponding sequence portion) complementary to the nucleic acid sequence of Figures 3b and 3c or Figures 10a and 10b, and/or 3) a sequence which encodes a product (e.g., a polypeptide or peptide) having the same functional characteristics of the product encoded by the sequence (or corresponding sequence portion) of the nucleic acid sequence of

SUBSTITUTE SHEET

- 22 -

Figures 3b and 3c or Figures 10a and 10b. Whether a functional equivalent must meet one or more criteria will depend on its use (e.g., if it is to be used only as an oligoprobe, it need meet only the first or second criteria and if it is to be used to produce *Lol p Ib*, it need only meet the third criterion).

A *Lol p Ib* cDNA, including *Lol p Ib.1* or *Lol p Ib.2* (or the mRNA from which it was transcribed) or a portion thereof can be used to identify similar sequences in any variety or type of plant and thus, to identify or "pull out" sequences which have sufficient homology to hybridize to *Lol p Ib* cDNA or mRNA or portion thereof, for example, DNA from allergens of plants of the family *Poaceae*, under conditions of low stringency. Those sequences which have sufficient homology (generally greater than 40%) can be selected for further assessment using the method described herein. Alternatively, high stringency conditions can be used. In this manner, DNA of the present invention can be used to identify, in other types of plants, preferably related families, genera, or species, sequences encoding polypeptides having amino acid sequences similar to that of *Lol p Ib* and thus to identify allergens in other species. Thus, the present invention includes not only *Lol p Ib*, but also other allergens encoded by DNA which hybridizes to DNA of the present invention.

The invention further includes isolated allergenic proteins or fragments thereof that are immunologically related to *Lol p Ib*, including *Lol p Ib.1* or *Lol p Ib.2* or fragments, or derivatives or homologues thereof, such as by antibody cross-reactivity wherein the isolated allergenic proteins or fragments thereof are capable of binding to antibodies specific for the protein and peptides of the invention, or by T cell cross-reactivity wherein the isolated allergenic proteins or fragments thereof are capable of stimulating T cells specific for the protein and peptides of this invention.

Work by others has shown that high doses of allergens generally produce the best results (i.e., best symptom relief). However, many people are unable to tolerate large doses of allergens because of allergic reactions to the allergens.

SUBSTITUTE SHEET

- 23 -

Modification of naturally- occurring allergens can be designed in such a manner that modified peptides or modified allergens which have the same or enhanced therapeutic properties as the corresponding naturally-occurring allergen but have reduced side effects (especially anaphylactic reactions) can be produced. These can be, for example, a protein or peptide of the present invention (e.g., one having all or a portion of the amino acid sequence of *Lol p Ib*, including *Lol p Ib.1* or *Lol p Ib.2* or purified native *Lol p Ib*), or a modified protein or peptide, or protein or peptide analogue. It is possible to modify the structure of a protein or peptide of the invention for such purposes as increasing solubility, enhancing therapeutic or preventive efficacy or stability (e.g., shelf life *ex vivo* and resistance to proteolytic degradation *in vivo*).

A modified protein or peptide can be produced in which the amino acid sequence has been altered, such as by amino acid substitution, deletion or addition, to modify immunogenicity and/or reduce allergenicity or to which a component has been added for the same purpose.

Thus, the present invention provides modified ryegrass pollen protein allergens which, when administered to a ryegrass pollen-sensitive individual, reduce the allergic response of the individual to ryegrass pollen. Preferred modified ryegrass pollen protein allergens include modified *Lol p Ib.1* protein or derivative or homologue thereof and modified *Lol p Ib.2* protein or derivative or homologue thereof. The present invention also provides at least one modified fragment of ryegrass pollen protein allergen which, when administered to a ryegrass pollen-sensitive individual, reduces the allergic response of the individual to ryegrass pollen. Preferably such modified fragments are at least one modified fragment of *Lol p Ib.1* protein or derivative or homologue thereof, or *Lol p Ib.2* protein or derivative or homologue thereof.

Lol p Ib.1 or *Lol p Ib.2* or purified native *Lol p Ib* protein or peptides can be modified, for example, using the polyethylene glycol method of A. Sehon and co-workers. Wie et al. (1981) *Int. Arch. Allergy Appl. Immunology*. 64: 84-99.

SUBSTITUTE SHEET

- 24 -

Modification of Lol p Ib.1 or Lol p Ib.2 or purified native Lol p Ib protein or peptides can also include reduction/alkylation (Tarr [1986] in: *Methods of Protein Microcharacterization*, J.E. Silver, ed. Humana Press, Clifton, NJ, pp 155-194); acylation (Tarr, *supra*); esterification (Tarr, *supra*); chemical coupling to an appropriate carrier (Mishell and Shiigi, eds, [1980] *Selected Methods in Cellular Immunology*, WH Freeman, San Francisco, CA; U.S. patent 4,939,239); or mild formalin treatment (Marsh [1971] *Int. Arch. Allergy Appl. Immunol.* 41: 199-215).

Another example of a modification of protein or peptides is substitution of cysteine residues preferably with alanine, serine, threonine, leucine or glutamic acid to minimize dimerization via disulfide linkages. Another example of modification of the peptides of the invention is by chemical modification of amino acid side chains or cyclization of the peptide.

In order to enhance stability and/or reactivity, the protein or peptides of the invention can also be modified to incorporate one or more polymorphisms in the amino acid sequence of the protein allergen resulting from natural allelic variation. Additionally, D-amino acids, non-natural amino acids or non-amino acid analogues can be substituted or added to produce a modified protein or peptide within the scope of this invention.

Native Lol p Ib may be purified using conventional methods known in the art such as those in Scopes, R.K. (1987), *Protein Purification, Principles and Practice*, Second Edition, Springer-Verlag, New York, New York. Suitable methods include ion exchange chromatography, high pressure liquid chromatography, electrophoresis, ultrafiltration, iso-electric-focusing and immunoabsorption chromatography using antibodies specific for native Lol p Ib. Purification of native Lol p Ib by iso-electric-focusing and SDS- PAGE is described in Example 2.

The cloning of the cDNAs encoding Lol p Ib.1 and Lol p Ib.2 was based on the recognition of the protein expressed

SUBSTITUTE SHEET

- 25 -

by Escherichia coli transformed with lambda-gt 11 phage, using both specific monoclonal antibodies and specific serum IgE from grass pollen-sensitive patients. Two such clones are designated 12R and 19R. Also, monoclonal antibodies used were

5 MAbs 3.2, FMC A7 (12.3), 21.3 and FMC A1 (40.1) (Kahn & Marsh (1986) *Molec. Immunol.* 23: 1281-1288; Singh & Knox (1985) *International Archives of Allergy and Applied Immunology* 78, 300-304; Smart et al. (1983) *International Archives of Allergy and Applied Immunology* 72 243-248). Details of the cloning of

10 *Lol p Ib.1* and *Lol p Ib.2* are given in the Examples.

The allergenic nature of the subject proteins are characterized in part, by their binding of the reaginic IgE antibodies which are present at high levels in sera of allergic patients. The IgE binding to the epitopes on allergic proteins

15 can be tested in a chromogenic assay in which allergens immobilized on a solid support can be visualized by sequential incubation in (1) allergic patients serum; (2) enzyme-labelled anti-IgE antibodies.

Another aspect of the present invention relates to

20 recombinant vectors comprising DNA sequences encoding proteins displaying allergenic activity from pollen of a grass species. More particularly, the grass species belongs to the family Poaceae (Gramineae), and even more particularly, to the genus *Lolium*. Still even more particularly, the allergenic protein

25 is characterized as being immunologically cross-reactive with antibody to *Lol p Ib* protein of *Lolium perenne* pollen, namely:

Pooid (festucoid) grasses. GROUP 1: Triticaneae: *Bromus inermis*, smooth broom; *Agropyron repens*, English couch; *A.cristatum*; *Secale cereale* rye *Triticum aestivum*, wheat.

30 GROUP 2: Poanae: *Dactylis glomerata*, orchard grass of cocksfoot; *Festuca elatior*, meadow fescue; *Lolium perenne*, perennial ryegrass; *L.multiflorum*, Italian ryegrass; *Poa pratensis*, Kentucky bluegrass; *P.compressa*, flattened meadow grass; *Avena sativa*, oat; *Holcus lanatus*, velvet grass or

35 Yorkshire fog; *Anthoxanthum odoratum*; sweet vernal grass; *Arrhenatherum elatius*, oat grass; *Agrostis alba*, red top; *Phleum pratense*, timothy; *Phalaris arundinacea*, reed canary

SUBSTITUTE SHEET

- 26 -

grass. Panicoid grass, *Paspalum notatum*, Bahia grass, Andropogonoid grasses: *Sorghum halepensis*, Johnson grass.

A variety of expression vectors can be constructed for the production of *Lol p Ib*, at least one fragment thereof or
5 their derivatives. Thus, a further aspect of the present invention provides recombinant vectors comprising DNA sequences encoding the allergenic protein *Lol p Ib* of ryegrass, *Lolium perenne*, *L. pollen*, or derivatives or homologues thereof. More particularly, the present invention relates to recombinant DNA
10 molecules comprising a eukaryotic or prokaryotic origin of replication, a detectable marker, DNA sequences encoding *Lol p Ib* family members or derivatives or homologues thereof, or allergenic proteins cross-reactive with antibodies to *Lol p Ib* family members or derivatives or homologues thereof, and,
15 optionally, promoter sequences capable of directing transcription of *Lol p Ib* family members.

The present invention also extends to the promoter of ryegrass pollen proteins, and particularly, to a promoter of a *Lol p Ib* gene such as the gene encoding *Lol p Ib.1* and *Lol p*
20 *Ib.2*. This promoter developmentally regulates a *Lol p Ib* gene expression and is organ, i.e., pollen specific. Developmental regulation as used herein refers to the expression of a particular trait, in this case allergenic proteins in pollen, during a certain stage in a plants life cycle and
25 non-expression during another stage. Hence, the *Lol p Ib* promoter is particularly useful in allowing expression of *Lol p Ib*, or any other gene or nucleotide sequence relative thereto, only during the development of pollen. The skilled artisan will immediately recognize the importance of such
30 promoters in selectively expressing a particular trait during pollen formation.

Accordingly, the present invention provides a method of inhibiting pollen development or function and thereby inducing nuclear male sterility in plants of the family
35 Poaceae, and in particular *Lolium perenne* L., comprising the steps of:

SUBSTITUTE SHEET

- 27 -

a) developing a plant carrying a recombinant DNA molecule comprising a ryegrass pollen promoter sequence or homologue or degenerate form thereof located on said molecule and a nucleotide sequence encoding a polypeptide having a deleterious function in cells derived from the family *Poaceae*, said nucleotide sequence transcribeable from said promoter, and said recombinant DNA molecule stably contained in pollen producing cells, and,

b) growing said plants under conditions and for a time sufficient for their development stage to cause expression of said nucleotide sequence from said promoter thereby producing the polypeptide having a deleterious function in said pollen producing cells such that pollen formation is inhibited or said pollen is inactive.

Well established methods exist for introducing recombinant DNA molecules into plant cells such as use of *Agrobacterium* plasmids and electroporation amongst others. By "deleterious function" in respect of a polypeptide refers to a feature of said polypeptide that will inhibit cell growth, cause lysis of a cell, or inhibit various functions in a cell and thereby prevent the normal functioning of the cell. In this case, lethal gene constructs having a deleterious function are contemplated which inhibit or prevent pollen formation and thereby result in a male sterile plant. Such "lethal genes" may encode enzymes, enzyme inhibitors, and/or toxic polypeptides, amongst other molecules. Alternatively, the lethal gene may encode an antisense RNA capable of inhibiting translation of a particular species of mRNA, the translated product thereof, being vital for pollen development.

Male sterile plants are particularly useful in developing hybrid crop varieties.

The *Lol p Ib* promoter is isolatable from ryegrass genomic DNA by any number of procedures including use of promoter probes vectors, "chromosome walking" and S1 nuclease mapping and sequencing as DNA upstream of the transcription initiation site.

Accordingly, the present invention provides a

SUBSTITUTE SHEET

- 28 -

recombinant DNA molecule comprising a ryegrass pollen promoter sequence, and in particular a promoter for a gene encoding a *Lol p Ib* family member, or homologues or degenerate forms thereof located on said molecule and further having one or more
5 restriction endonuclease sites downstream of said promoter such that a nucleotide sequence inserted into one or more of these sites is transcribable in the correct reading frame and is thereby a developmentally regulated, pollen-specific expression vector. As used herein, the "correct reading frame" has the
10 same meaning as "in phase". The aforementioned DNA molecule will preferably also have a selectable marker thereon, such as an antibiotic or other drug resistance gene, such as for example gene encoding resistance to ampicillin, carbenicillin, tetracycline, streptomycin and the like. The recombinant
15 molecule will further comprise a means for stable inheritance in a prokaryotic and/or eukaryotic cell. This can be accomplished by said recombinant molecule carrying a eukaryotic and/or a prokaryotic origin of replication as hereinbefore described in relation to expression vectors.

20 Alternatively, the recombinant molecule will carry a means for integration into a host cell genome thereby permitting replication of said recombinant molecule in synchrony with the replication of said host cell genome. Examples of preferred prokaryotic hosts include cells *E. coli*,
25 *Bacillus* and *Pseudomonas* amongst others. Preferred eukaryotic hosts include cells from yeast and fungi, insects, mammals and plants. Even more preferred host cells are plants of the family *Poaceae*, and in particular of the genus *Lolium*, such as *Lolium perenne*. Accordingly in a preferred embodiment, a *Lol*
30 *p Ib* gene promoter with a gene encoding a deleterious function positioned relative thereto will be carried by a recombinant DNA molecule capable of integration into the genome of cells of plants from the family *Poaceae*, or *L. perenne*. Such a recombinant DNA molecule is transferred to the aforementioned
35 cells by, for example, electroporation. Ideally, said cells are callus-derived cells. Said callus-derived cells transformed with said recombinant DNA molecule are then

SUBSTITUTE SHEET

- 29 -

permitted to regenerate into whole plants. Whole plants entering the pollen development stage of its like cycle permit functioning of a *Lol p Ib* gene promoter and, hence, expression of the gene encoding a deleterious function. Consequently, pollen development is inhibited or prevented and a nuclear male sterile plant results therefrom.

Alternatively, a *Lol p Ib* promoter will direct expression of a gene having advantageous functions, such as a cytokinin. All such recombinant DNA molecules are encompassed by the present invention.

The present invention extends to monoclonal and polyclonal antibodies to *Lol p Ib* or at least one fragment of recombinantly or synthetically produced *Lol p Ib* or purified native *Lol p Ib*, produced according to the methods described in International Patent Application No. PCT/AU89/00123 and to their use in immunoassays and test kits as described therein.

The monoclonal antibodies used in the present work to screen the cDNA library for *Lol p Ib* clones showed cross-reactivity with allergenic proteins from pollen of various related grass species. This shows there is a homology between allergenic proteins produced by these pollens with *Lol p Ib* protein allergens supporting the applicability of the present invention to all related grasses. The present invention also relates to antibodies to recombinant *Lol p Ib* protein allergens and derivatives, homologues and immunological relatives thereof including chemical synthetic derivatives thereof. In the following discussion, reference to *Lol p Ib* protein allergens includes its derivatives, homologues and immunological relatives and chemical synthetic derivatives thereof. The following discussion also includes antibodies specific for purified *Lol p Ib* and fragments, derivative and homologues thereof. Such antibodies are contemplated to be useful in developing detection assays (immunoassays) for *Lol p Ib* protein allergens especially during the monitoring of a therapeutic or diagnostic regimen and in the purification of recombinantly or synthetically produced *Lol p Ib* family members or purified native *Lol p Ib*. The antibodies may be monoclonal

SUBSTITUTE SHEET

- 30 -

or polyclonal. Additionally, it is within the scope of this invention to include any second antibodies (monoclonal or polyclonal) directed to the first antibodies discussed above. The present invention further contemplates use of these first
5 or second antibodies in detection assays and, for example, in monitoring the effect of a diagnostic or an administered pharmaceutical preparation. Furthermore, it is within the scope of the present invention to include antibodies to any molecules complexed with a Lol p Ib protein allergen.
10 Accordingly, an antibody to a Lol p Ib protein allergen encompasses antibodies to such Lol p Ib protein allergen, or antigenic parts thereof, and to any associated molecules (e.g., lipid regions, carrier molecules, fused proteins, and the like).

15 The Lol p Ib family members, or fragments thereof, considered herein are purified then utilized in antibody production. Both polyclonal and monoclonal antibodies are obtainable by immunization with recombinant, synthetic or native Lol p Ib protein family members, and either type is
20 utilizable for immunoassays. The methods of obtaining both types of sera are well known in the art. Polyclonal sera are less preferred but are relatively easily prepared by injection of a suitable laboratory animal with an effective amount of a purified Lol p Ib family member, or antigenic parts thereof,
25 collecting serum from the animal, and isolating specific sera by any of the known immunoabsorbent techniques. Although antibodies produced by this method are utilizable in virtually any type of immunoassay, they are generally less favored because of the potential heterogeneity of the product.

30 The use of monoclonal antibodies in an immunoassay is particularly preferred because of the ability to produce them in large quantities and the homogeneity of the product. The preparation of hybridoma cell lines for monoclonal antibody production derived by fusing an immortal cell line and
35 lymphocytes sensitized against the immunogenic preparation can be done by techniques which are well known to those who are skilled in the art. (See, for example, Kohler and Milstein

SUBSTITUTE SHEET

- 31 -

(1975) *Nature* 256: 495-499, and Kohler and Milstein (1986) *Eur. J. Immunol.* 6: 511-519.

Unlike preparation of polyclonal sera, the choice of animal is dependent on the availability of appropriate immortal
5 lines capable of fusing with lymphocytes. Mouse and rat have been the animals of choice in hybridoma technology and are preferably used. Humans can also be utilized as sources for sensitized lymphocytes if appropriate immortalized human (or nonhuman) cell lines are available. For the purpose of the
10 present invention, the animal of choice may be injected with from about 0.1 mg to about 20 mg of purified recombinant or native Lol p Ib, or parts thereof. Usually the injecting material is emulsified in Freund's complete adjuvant. Boosting injections may also be required. The detection of antibody
15 production can be carried out by testing the antisera with appropriately labelled antigen. Lymphocytes can be obtained by removing the spleen or lymph nodes of sensitized animals in a sterile fashion and carrying out fusion. Alternatively, lymphocytes can be stimulated or immunized *in vitro*, as
20 described, for example, in Reading (1982) *J. Immunol. Methods* 53:261-291.

A number of cell lines suitable for fusion have been developed, and the choice of any particular line for hybridization protocols is directed by any one of a number of
25 criteria such as speed, uniformity of growth characteristics, deficiency of its metabolism for a component of the growth medium, and potential for good fusion frequency.

Intraspecies hybrids, particularly between like strains, work better than interspecies fusions. Several cell
30 lines are available, including mutants selected for the loss of ability to secrete myeloma immunoglobulin.

Cell fusion can be induced either by virus, such as Epstein-Barr or Sendai virus, or polyethylene glycol. Polyethylene glycol (PEG) is the most efficacious agent for the
35 fusion of mammalian somatic cells. PEG itself may be toxic for cells, and various concentrations should be tested for effects on viability before attempting fusion. The molecular weight

SUBSTITUTE SHEET

- 32 -

range of PEG may be varied from 1000 to 6000. It gives best results when diluted to from about 20% to about 70% (w/w) in saline or serum-free medium. Exposure to PEG at 37°C for about 30 seconds is preferred in the present case, utilizing murine
5 cells. Extremes of temperature (i.e., about 45°C) are avoided, and preincubation of each component of the fusion system at 37°C prior to fusion can be useful. The ratio between lymphocytes and malignant cells is optimized to avoid cell fusion among spleen cells and a range of from about 1:1 to
10 about 1:10 is commonly used.

The successfully fused cells can be separated from the myeloma line by any technique known by the art. The most common and preferred method is to chose a malignant line which is hypoxanthine guanine phosphoribosyl transferase (HGPRT)
15 deficient, which will not grow in an aminopterin-containing medium used to allow only growth of hybrids, and aminopterin-containing medium used to allow only growth of hybrids and which is generally composed of hypoxanthine $1.10^{-4}M$, aminopterin $1 \times 10^{-5}M$, and thymidine $3 \times 10^{-5}M$, commonly known as
20 the HAT medium. The fusion mixture can be grown in the HAT-containing culture medium immediately after the fusion or 24 hours later. The feeding schedules usually entail maintenance in HAT medium for two weeks and then feeding with either regular culture medium or hypoxanthine,
25 thymidine-containing medium.

The growing colonies are then tested for the presence of antibodies that recognize the antigenic preparation. Detection of hybridoma antibodies can be performed using an assay where the antigen is bound to a solid support and allowed
30 to react to hybridoma supernatants containing putative antibodies. The presence of antibodies may be detected by "sandwich" techniques using a variety of indicators. Most of the common methods are sufficiently sensitive for use in the range of antibody concentrations secreted during hybrid growth.

35 Cloning of hybrids can be carried out after 21- 23 days of cell growth in selected medium. Cloning can be preformed by cell limiting dilution in fluid phase or by

SUBSTITUTE SHEET

- 33 -

directly selecting single cells growing in semi-solid agarose. For limiting dilution, cell suspensions are diluted serially to yield a statistical probability of having only one cell per well. For the agarose technique, hybrids are seeded in a semisolid upper layer, over a lower layer containing feeder cells. The colonies from the upper layer may be picked up and eventually transferred to wells.

Antibody-secreting hybrids can be grown in various tissue culture flasks, yielding supernatants with variable concentrations of antibodies. In order to obtain higher concentrations, hybrids may be transferred into animals to obtain inflammatory ascites. Antibody-containing ascites can be harvested 8-12 days after intraperitoneal injection. The ascites contain a higher concentration of antibodies but include both monoclonals and immunoglobulins from the inflammatory ascites. Antibody purification may then be achieved by, for example, affinity chromatography.

The presence of Lol p Ib protein allergen contemplated herein, or antibodies specific for same, in a patient's serum, plant or mammalian tissue or tissue extract, can be detected utilizing antibodies prepared as above, either monoclonal or polyclonal, in virtually any type of immunoassay. A wide range of immunoassay techniques are available as can be seen by reference to U.S. Patent No. 4,015,043, 4,424,279 and 4,018,653. This, of course, includes both single-site and two-site, or "sandwich", assays of the non-competitive types, as well as in the traditional competitive binding assays. Sandwich assays are among the most useful and commonly used assays and are favored for use in the present invention. A number of variations of the sandwich assay technique exist, and all are intended to be encompassed by the present invention. Briefly, in a typical forward assay, an unlabeled antibody is immobilized in a solid substrate and the sample to be tested brought into contact with the bound molecule. After a suitable period of incubation, for a period of time sufficient to allow formation of an antibody-antigen secondary complex, a second antibody, labelled with a reporter molecule capable of

SUBSTITUTE SHEET

- 34 -

producing a detectable signal is then added and incubated, allowing time sufficient for the formation of a tertiary complex of antibody-antigen- labelled antibody (e.g., antibody-Lol p Ib protein-antibody). Any unreacted material is washed away, and the presence of the antigen is determined by observation of a signal produced by the reporter molecule. The results may either be qualitative, by simple observation of the visible signal, or may be quantitated by comparing with a control sample containing known amounts of hapten. Variations on the forward assay include a simultaneous assay, in which both sample and labelled antibody are added simultaneously to the bound antibody, or a reverse assay in which the labelled antibody and sample to be tested are first combined, incubated and then added simultaneously to the bound antibody. These techniques are well known to those skilled in the art, including any minor variations as will be readily apparent.

Although the following discussion is concerned with detecting Lol p Ib, it is equally applicable to detecting antibodies to Lol p Ib and it is intended to be a sufficient description thereof. In the typical forward sandwich assay, a first antibody having specificity for Lol p Ib, or antigenic parts thereof, contemplated in this invention, is either covalently or passively bound to a solid surface. The solid surface is typically glass or a polymer, the most commonly used polymers being cellulose, polyacrylamide, nylon, polystyrene, polyvinyl chloride or polypropylene. The solid supports may be in the form of tubes, beads, discs of microplates, or any other surface suitable for conducting an immunoassay. The binding processes are well-known in the art and generally consist of cross-linking covalently binding or physically adsorbing, the polymer-antibody complex is washed in preparation for the test sample. An aliquot of the sample to be tested is then added to the solid phase complex and incubated at 25°C for a period of time sufficient to allow binding of any subunit present in the antibody. The incubation period will vary but will generally be in the range of about 2-40 minutes. Following the incubation period, the antibody subunit solid phase is washed

SUBSTITUTE SHEET

- 35 -

and dried and incubated with a second antibody specific for a portion of the hapten. The second antibody is linked to a reporter molecule which is used to indicate the binding of the second antibody to the hapten.

5 By "reporter molecule," as used in the present specification, is meant a molecule which, by its chemical nature, provides an analytically identifiable signal which allows the detection of antigen-bound antibody. Detection may be either qualitative or quantitative. The most commonly used
10 reporter molecules in this type of assay are either enzymes, fluorophores or radionuclide containing molecules (i.e., radioisotopes). In the case of an enzyme immunoassay, an enzyme is conjugated to the second antibody, generally by means of glutaraldehyde or periodate. As will be readily recognized,
15 however, a wide variety of different conjugation techniques exist, which are readily available to the skilled artisan. Commonly used enzymes include horseradish peroxidase, glucose oxidase, beta-galactosidase and alkaline phosphatase, amongst others. The substrates to be used with the specific enzymes
20 are generally chose for the production, upon hydrolysis by the corresponding enzyme, of a detectable color change. For example, p-nitrophenyl phosphate is suitable for use with alkaline phosphatase conjugates; for peroxidase conjugates, 1,2- phenylenediamine, 5-aminosalicylic acid, or toluidine are
25 commonly used. It is also possible to employ fluorogenic substrates, which yield a fluorescent product rather than the chromogenic substrates noted above. In all cases, the enzyme-labelled antibody is added to the first antibody hapten complex, allowed to bind, and then the excess reagent is washed
30 away. A solution containing the appropriate substrate is then added to the tertiary complex of antibody-antigen-antibody. The substrate will react with the enzyme linked to the second antibody, giving a qualitative visual signal, which may be further quantitated, usually spectrophotometrically, to give an
35 indication of the amount of hapten which was present in the sample. "Reporter molecule" also extends to use of cell agglutination or inhibition of agglutination such as red blood

SUBSTITUTE SHEET

- 36 -

cells or latex beads, and the like.

Alternately, fluorescent compounds, such as fluorescein and rhodamine, may be chemically coupled to antibodies without altering their binding capacity. When activated by illumination with light of a particular wavelength, the fluorochrome-labelled antibody adsorbs the light energy, inducing a state of excitability in the molecule, followed by emission of the light at a characteristic color visually detectable with a light microscope. As in the EIA, the fluorescent labelled antibody is allowed to bind to the first antibody-hapten complex. After washing off the unbound reagent, the remaining tertiary complex is then exposed to the light of the appropriate wavelength, the fluorescein observed indicates the presence of the hapten of interest. Immunofluorescence and EIA techniques are both very well established in the art and are particularly preferred for the present method. However, other reporter molecules, such as radioisotope, chemilluminiscent or bioluminescent molecules, may also be employed. It will be readily apparent to the skilled technician how to vary the procedure to suit the required purpose. It will also be apparent that the foregoing can be used to detect directly or indirectly (i.e., via antibodies) Lol p Ib protein of this invention.

Accordingly, one aspect of the present invention provides a method of detecting Lol p Ib or a derivative or homologue thereof or an allergenic protein immunologically reactive with said Lol p Ib or derivatives or homologues present in serum, tissue extract, plant extract or other biological fluid comprising the steps of containing said serum, extract or fluid to be tested with an antibody to said Lol p Ib protein for a time and under conditions sufficient for an allergenic protein-antibody complex to form and subjecting said complex to a detecting means. The present invention also provides a method of detecting an antibody to an allergenic protein from pollen of the family Poaceae (Gramineae) in serum or other biological fluid comprising contacting said serum or fluid with a Lol p Ib protein, or derivative or homologue

SUBSTITUTE SHEET

- 37 -

thereof, or its antigenic derivative for a time and under conditions sufficient for an antibody-Lol p Ib complex to form and subjecting said complex to a detecting means. The latter complex may be detected by the Lol p Ib protein having attached thereto a reporter molecule or by addition of a second antibody labelled with a reporter molecule.

Accordingly, the present invention is also directed to a kit for the rapid and convenient assay for antibodies to Lol p Ib or derivatives, homologues or immunological relatives thereof in mammalian body fluids (e.g., serum, tissue extracts, tissue fluids), in vitro cell culture supernatants, and cell lysates. The kit is compartmentalized to receive a first container adapted to an antigenic component thereof, and a second container adapted to contain an antibody to Lol p Ib, said antibody being labelled with a reporter molecule capable of giving a detectable signal as hereinbefore described. If the reporter molecule is an enzyme, then a third container adapted to contain a substrate for said enzyme is provided. In an exemplified use of the subject kit, a sample to be tested is contacted with the contents of the first container for a time and under conditions for an antibody, if present in the sample, to bind to Lol p Ib protein in said first container. If the Lol p Ib protein of the first container has bound to antibodies in the test fluid, the antibodies of the second container will bind to the secondary complex to form a tertiary complex and, since these antibodies are labelled with a reporter molecule, when subjected to a detecting means, the tertiary complex is detected. Therefore, one aspect of the present invention is a kit for the detection of antibodies to a protein having allergenic properties, said protein from pollen of the family Poaceae (Gramineae), the kit being compartmentalized to receive a first container adapted to contain recombinant Lol p Ib protein or its antigenic derivative or homologue or a purified native Lol p Ib protein or its antigenic derivative or homologue, and a second container adapted to contain an antibody to Lol p Ib or derivative or homologue thereof, said antibody labelled with a reporter molecule capable of giving a

SUBSTITUTE SHEET

- 38 -

detectable signal. The "reporter molecule" may also involve agglutination of red blood cells (RBC) on latex beads. In this kit the reporter molecule is a radioisotope, an enzyme, an fluorescent molecule, a chemilluminescent molecule, bioluminescent molecule or RBC. The kit alternatively comprises a container adapted to contain recombinant *Lol p Ib* or is antigenic derivative or homologue labelled with a reporter molecule capable of giving a detectable signal.

Because of the presence of allergens in the environment, hayfever and seasonal asthma continue to have significant morbidity and socio-economic impact on Western communities, despite advances made in their pharmacology and immunology. While the available spectrum of drugs, including anti-histamines and steroids have resulted in improvement in the treatment of allergic disease, they have unfortunate side-effects associated with long-term usage. Because of these problems, renewed interest has been shown in the immunotherapy of allergic disease. Immunotherapy involves the injection of potent allergen extracts to desensitize patients against allergic reactions (Bousquet, & Michel (1989) *Allergy Clin. Immunol. News* 1: 7-10). Unfortunately, the pollen preparations used as allergens are polyvalent and of poor quality. Consequently, concentrations used are frequently high in order to induce IgG responses, but may be lethal through triggering of systemic reactions, including anaphylaxis. The cloned gene product or synthetic peptides based on the sequence of allergens provides a safer medium for therapy since it can be quality controlled, characterized and standardized.

The precise mechanism for symptomatic relief remains hypothetical. However, administration of a preparation comprising recombinant, synthetic or purified native *Lol p Ib* or at least one antigenic fragment thereof, of the instant invention to a ryegrass sensitive individual will modify the allergic response of a ryegrass sensitive individual to ryegrass pollen allergens, e.g. by modifying the B-cell response to *Lol p Ib*, the T-cell response to *Lol p Ib*, or both the B cell and T cell response to *Lol p Ib*.

SUBSTITUTE SHEET

- 39 -

Currently immunotherapy is one of the most frequently administered treatments in allergology, and in the USA it is considered the first choice. An advantage of this treatment for pollen rhinitis is that treatment takes up to 3 years, while pharmacotherapy must be carried out during the patient's entire life time. Patients given pollen extract for immunotherapy showed a clinical benefit that lasted for four years after the end of treatment (Grammer et al. (1984) *J. Allergy Clin. Immunol* 73: 484- 489).

Immune responsiveness to rye-grass pollen allergens *Lol p* II and *Lol p* III in the human population is significantly associated with the histocompatibility leukocyte antigen HLA-DR3 (Friedhoff et al. (1988) *Tissue Antigens* 31: 211-219; Ansari, et al. (1989) *Human Immunol.* 25: 59-71; Ansari et al. (1989) *Int. Arch. Allergy Appl. Immunol.* 88: 164-189). This means that the HLA-DR3 encoded class II Ia molecules of the antigen-presenting cells may recognize a similar immunodominant T cell/Ia recognition site present on another allergen. *Lol p* Ia is known to share an immunodominant T cell/Ia recognition site (YTTEGGTKS EVEDV IP) with both *Lol p* II and *Lol p* III (Friedhoff et al., supra). Most allergic individuals who respond to *Lol p* II and III also respond to *Lol p* Ia, but not the reciprocal. Thus, *Lol p* Ia appears to have unique T cell/Ia recognition site(s) not present in *Lol p* II or III. Furthermore, the common T cell/Ia recognition site shared between *Lol p* Ia, II and III is not represented in the deduced sequence of *Lol p* Ib.1 or *Lol p* Ib.2.

Accordingly, the present invention is directed to the *Lol p* Ib family of protein allergens, their derivatives, homologues or immunological relatives including derivatives containing the common antigenic epitope between *Lol p* Ia and *Lol p* Ib.1 which are useful in developing a vaccine to desensitize humans to allergies due to grass pollen.

Accordingly, the present invention provides a method for desensitizing a human allergic to grass pollen which comprises administering to said human a desensitizing-effective amount of *Lol p* Ib or at least one fragment or a derivative,

SUBSTITUTE SHEET

- 40 -

homologue, or immunological relative thereof, for a time and under conditions sufficient to effect desensitization of the human to the grass pollen.

The present invention also provides a method of
5 treating sensitivity to ryegrass pollen in a mammal sensitive to such pollen, comprising administering to the mammal a therapeutically effective amount of a therapeutic composition of the invention. The present invention further provides a method of treating sensitivity to ryegrass pollen allergen or
10 an allergen immunologically cross-reactive with ryegrass pollen allergen comprising administering to a mammal a therapeutically effective amount of said protein preparation of the invention.

Through the use of the peptides and protein of the present invention, preparations of consistent, well-defined
15 composition and biological activity can be made and administered for therapeutic purposes (e.g., to modify the allergic response of a *L perenne* sensitive individual to pollen of such plants. Administration of such peptides or protein may, for example, modify B-cell response to *Lol p Ib* allergen,
20 T-cell response to *Lol p Ib* allergen, or both responses. Purified peptides can also be used to study the mechanism of immunotherapy of *L. perenne* allergy and to design modified derivatives or analogues useful in immunotherapy.

The present invention, therefore, provides a
25 pharmaceutical compositions comprising a desensitizing or therapeutically effective amount of *Lol p Ib* or derivatives, homologues or immunological relatives thereof and one or more pharmaceutically acceptable carriers and/or diluents. The active ingredients of a pharmaceutical composition comprising
30 *Lol p Ib* is contemplated to exhibit excellent therapeutic activity, for example, in the desensitization of humans allergic to grass pollen when administered in amount which depends on the particular case. For example, from about 0.5 ug to about 20 mg per kilogram of body weight per day may be
35 administered. Dosage regime may be adjusted to provide the optimum therapeutic response. For example, several divided doses may be administered daily or the dose may be

SUBSTITUTE SHEET

- 41 -

proportionally reduced as indicated by the exigencies of the therapeutic situation. The active compound may be administered in a convenient manner such as by the oral, intravenous (where water soluble), intramuscular, subcutaneous, intranasal, 5 intradermal or suppository routes or implanting (e.g., using slow release molecules). Depending on the route of administration, the active ingredients which comprise the pharmaceutical composition of the invention may be required to be coated in a material to protect said ingredients from the 10 action of enzymes, acids and other natural conditions which may inactivate said ingredients. For example, Lol p Ib may be administered in an adjuvant, co-administered with enzyme inhibitors or in liposomes. Adjuvant is used in its broadest sense and includes any immune stimulating compound, such as 15 interferon. Adjuvants contemplated herein include resorcinols, non-ionic surfactants such as polyoxyethylene oleyl ether and n-hexadecyl polyethylene ether. Enzyme inhibitors include pancreatic trypsin. Liposomes include water-in-oil-in-water CGF emulsions as well as conventional liposomes. For purposes of 20 inducing T cell anergy, the pharmaceutical composition if preferably administered in non-immunogenic form (e.g. it does not contain adjuvant).

The active compounds may also be administered parenterally or intraperitoneally. Dispersions can also be 25 prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms.

The pharmaceutical forms suitable for injectable use 30 include sterile aqueous solutions (where water soluble) or dispersions and sterile powders of the extemporaneous dispersion. In all cases the form must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must 35 be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol,

SUBSTITUTE SHEET

- 42 -

polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. The preventions of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions are prepared by incorporating the active compounds in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and the freeze-drying technique which yield a powder of the active ingredient plus any additional desired ingredient from previously sterile-filtered solution thereof.

When at least one Lol p Ib family member, or at least one fragment thereof is suitably protected as described above, the active compound may be orally administered, for example, with an inert diluent or with an assimilable edible carrier, or it may be enclosed in hard or soft shell gelatin capsule, or it may be compressed into tablets, or it may be incorporated directly with food of the diet. For oral therapeutic administration, the active compound may be incorporated with excipients and used in the form of ingestible tablets, buccal tablets, troches, capsules, elixirs, suspensions, syrups,

SUBSTITUTE SHEET

- 43 -

wafers, and the like. Such compositions and preparations should contain at least 1% by weight of active compound. The percentage of the compositions and preparations may, of course, be carried and may conveniently be between about 5 to 80% of the weight of the unit. The amount of active compound in such therapeutically useful compositions is such that a suitable dosage will be obtained. Preferred compositions or preparations according to the present invention are prepared so that an oral dosage unit form contains between about 10 ug. and 2000 mg of active compound.

The tablets, troches, pills, capsules and the like may also contain the following: A binder such as gum tragacanth, acacia, corn starch or gelatin; excipients such as dicalcium phosphate; a disintegrating agent such as corn starch, potato starch, alginic acid and the like; a lubricant such as magnesium stearate; and a sweetening agent such as sucrose, lactose or saccharin may be added or a flavoring agent such as peppermint, oil of wintergreen, or cherry flavoring. When the dosage unit form is a capsule, it may contain, in addition to materials of the above type, a liquid carrier. Various other materials may be present as coatings or to otherwise modify the physical form of the dosage unit. For instance, tablets, pills, or capsules may be coated with shellac, sugar or both. A syrup or elixir may contain the active compound, sucrose as a sweetening agent, methyl and propylparabens as preservatives, a dye and flavoring such as cherry or orange flavor. Of course, any material used in preparing any dosage unit form should be pharmaceutically pure and substantially non-toxic in the amounts employed. In addition, the active compound may be incorporated into sustained-release preparations and formulations.

As used herein "pharmaceutically acceptable carrier and/or diluent" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutical active substances is well known in the art. Except insofar as any conventional media or

SUBSTITUTE SHEET

- 44 -

agent is incompatible with the active ingredient, use thereof in the therapeutic compositions is contemplated. Supplementary active ingredients can also be incorporated into the compositions.

5 It is especially advantageous to formulate parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the mammalian subjects to be treated; each unit containing a
10 predetermined quantity of active material calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the novel dosage unit forms of the invention are dictated by and directly dependent on (1) the unique characteristics of the active
15 material and the particular therapeutic effect to be achieved, and (b) the limitations inherent in the art of compounding such an active material for the treatment of disease in living subjects having a diseased condition in which bodily health is impaired as herein disclosed in detail.

20 The principal active ingredient is compounded for convenient and effective administration in effective amounts with a suitable pharmaceutically acceptable carrier in dosage unit form as hereinbefore disclosed. A unit dosage form can, for example, contain the principal active compound in amounts
25 ranging from about 10 µg to about 2000 mg. Expressed in proportions, the active compound is generally present in from about 10 µg to about 2000 mg/ml of carrier. In the case of compositions containing supplementary active ingredients, the dosages are determined by reference to the usual dose and
30 manner of administration of the said ingredients.

The present invention is further illustrated by the following non-limiting Figures and Examples.

EXAMPLES

Example 1 - Isolation of cDNA Clones

35 A cDNA expression library in the vector lambda-gt 11 was prepared from polyadenylated mRNA of mature rye-grass

SUBSTITUTE SHEET

- 45 -

pollen (Beall & Mitchell (1986) *J. Immunol. Methods* 86: 217-223). This library was screened initially with monoclonal antibody (Mab) FMC-A1 (40.1) (Fig. 1a).

Poly (A+) mRNA isolated from mature rye grass pollen
5 by the phenol method (Herrin and Michaels, (1984) *Plant Mol. Biol. Rep.*, 2:24-28) was used to construct a cDNA library in the vector lambda-gt 11. The library was then screened with antibody probes to detect sequences expressing Group I
10 proteins. *E. coli* Y1090 transfected with 3×10^4 recombinant phages were plated and incubated at 42°C for 3 h. The plates were overlaid with a dry 132 mm nitrocellulose (NC) filter previously soaked in 10mm IPTG and transferred to 37°C. After
15 incubation for 3 h the filters were carefully peeled off and incubated in 20 ml per filter of MTBS (10% w/v non-fat milk powder, 50 mM Tris-HCl, pH 7.6, 150 mM NaCl) for 30 min. at
20 room temperature. A second set of NC filters was placed on phage plates and after incubating for 3 h were treated as above. Both sets of NC filters were tested for binding of Mab 40.1 to plaques by the method described in Huynh et al. (1985)
25 In: DNA Cloning, a practical approach, Glover, D.M. (ed.) Vol. 1, pp. 49-78, IRL Press, Oxford, England. The antibody positive plaques were picked, purified, then replated and tested for binding to probes. The positive clones were
30 plaque-purified and tested for IgE binding using sera from grass pollen- allergic subjects. Eighteen clones were selected as encoding proteins recognized by both Lol p I-specific MABs and IgE antibodies (Table 1). The largest of the cDNA clones, 1.2kb in size, that expressed rye-grass allergenic protein was initially selected for further characterization and sequencing,
and designated clone lambda-12R (Fig. 1a).

SUBSTITUTE SHEET

- 46 -

Table 1
 Characteristics of cDNA Clones Expressing Group I
 Allergens of Ryegrass

	Clone No. (-R)	Binding of MAb 12.3 ^a	Binding of MAb 40.1 ^a	Binding of IgE from sera of allergic indivs.	Approx. Size of Insert (bp)
5	1	-	-	-	
10	2	+	++	-	700
	3	+	++	-	600
	4	+	++	-	800
	5	+	++	-	500
	6	+	++	-	600
15	7	+	++	-	400
	8	-	-	-	
	9	-	-	-	
	10	-	-	-	
	11	+	++	-	500
20	12	++	++	++	1200
	13	+	++	+	800
	14	++	++	++	1200
	15	-	-	-	
	16	+	++	-	800
25	17	+	++	-	400
	18	++	++	++	1200

++ : -strongest binding

- : -no binding

MAb 12.3 shows high affinity for Lol p Ib.1 encoded by clone

30 12R.

The specificity of IgE and MAbs was tested by immunoblot analysis of rye-grass pollen protein extracts (Fig. 1b).

Soluble proteins were extracted from rye-grass pollen by vigorous shaking in PBS (150 mM NaCl in 10 mM sodium phosphate, pH 7.2) on ice for 3 h. Pollen was spun out of solution and the extracted protein standardized using the Biorad assay. 120 ug protein per lane was electrophoresed under reducing conditions on a 10-15% w/v SDS-polyacrylamide gel. Proteins were electroblotted onto NC filters and the blot blocked with TBS (10 mM Tris, 150 mM NaCl, pH 7.9) containing 10% w/v non-fat milk powder. The blot was cut into strips and each treated with the various probes: MAbs were diluted 1:1000 in TBS containing 1% BSA. Sera collected from at least

SUBSTITUTE SHEET

- 47 -

4 patients with high RAST scores for grass pollen, was pooled and used diluted 1:5 in TBS/1% w/v BSA for IgE binding. Horseradish peroxidase-conjugated secondary antibodies were used (Dakopatts, Glostrup, Denmark) and after washing, binding was visualized with 4-chloro 1-naphthol (Biorad, Richmond, CA) and H_2O_2 .

When the immunoblot was incubated in pooled sera from grass pollen-allergic individuals, strong IgE binding was observed throughout the 28-35 kD region. The MAbs used in this study, 3.2, 12.3, 21.3 and 40.1 had previously been partially characterized (Kahn and Marsh (1986) *Molec. Immunol.* 23: 1281-1288; Singh and Knox (1985) *Intl. Arch. Allergy and Applied Immunol.* 78: 300-304; Smart et al. (1983) *Intl. Arch. Allergy and Applied Immunol.* 72: 243- 248). MAbs 3.2, 21.3 and 40.1 showed strong reactivity with the proteins in the 28-35 kD region. MAb 12.3 exhibited no binding to the 35 Kd band, but bound strongly to the lower bands. These interactions suggest that both IgE and MAbs can recognize denatured allergens, which makes them suitable probes for the detection of recombinant protein expressed in *E. coli*. It was previously thought that MAb FMC-A1 had a strong preference for Lol p Ia, although it would also bind to a lesser extent to Lol p Ib. New data suggests that the original FMC-A1 preparation may be polyclonal rather than monoclonal. One antibody in the FMC-A1 preparation appears to be specific for Lol p Ia while one appears to be specific for Lol p Ib. Therefore, the apparent cross-reaction of Lol p Ia and Lol p Ib defined by FMC-A1 may reflect polyclonality in this antibody preparation.

The allergen-beta-galactosidase fusion protein produced by the induction of lysogenic cultures of the lambda clone containing the 12R insert was characterized by immunoblot analysis using MAb 40.1. This fusion protein of approximately 146 kD is assumed to be comprised of the 116 kD beta-galactosidase and 30 kD of allergen-encoded sequence. This fusion protein was produced in low yields. So in order to increase yields of the cloned allergen for further analysis, we used an alternative expression system. The 1.2 kb insert was

SUBSTITUTE SHEET

- 48 -

subcloned in the pGEX1-3 series of plasmid expression vectors. These plasmids give a fusion polypeptide with the carboxyl terminus of the *Schistosoma japonicum* glutathione S-transferase protein (Smith and Johnson, (1988), Gene, 67:31-40). Strong
5 IgE binding was detected only in bacteria transformed with pGEX-12R, and not in those with parental pGEX plasmids (data not shown, but similar binding shown in Fig. 4). Probing of Western blots with control sera that had negative radioallergosorbent (RAST) score for rye-grass pollen showed no
10 IgE binding.

Example 2 - Identity of Cloned Allergen 12R

All four MABs used in this study recognized the cloned allergen 12R (Fig. 1a).

Not all MABs show the same specificity to the native
15 *Lol p I* proteins (Fig. 1b). In particular, MAB 12.3 does not recognize the 35 kD band. Because the cloned allergen binds all the MABs, and with high intensity to MAB 12.3, it is predicted that the cloned allergen is likely to correspond to a protein of lower Mr, and not to the 35 kD protein. To
20 confirm its identity, an immunological approach developed for parasite antigens was employed (eg Beall & Mitchell (1986) J. Immunol. Methods 86: 217-223). In this method, the cloned allergen 12R was immobilized on nitrocellulose membrane, and used to bind specific IgE antibodies from sera. Bound
25 antibodies were eluted and used to probe a Western blot of rye-grass pollen proteins. Highly specific and reproducible patterns of binding were consistently obtained in several experiments to two protein components of molecular weight 31 and 33 kD. The 35 kD band has been designated *Lol p Ia* and the
30 31 and 33 kD bands have been designated *Lol p Ib*. These experiments demonstrate that IgE antibodies that bind to clone 12R recognize two components with slightly different molecular weights, 31 and 33 kD. No specific binding was observed when IgE antibodies from non-grass pollen allergic individuals were
35 used nor when extracts of *E. coli* transformed with non-recombinant pGEX plasmids were used to select IgE

SUBSTITUTE SHEET

- 49 -

antibodies.

Lol p Ib protein was purified by two-dimensional analysis involving preparative iso-electric focusing in the first dimension, followed by SDS-PAGE of the individual
5 fractions collected. This procedure successfully separated *Lol p Ib* in sufficient quantity for the N-terminal sequence to be determined (Table 2).

Table 2
N-Terminal Amino Acid Sequences Of Grass Pollen Allergens
10 Obtained In This Study Compared With Reported Sequences

Allergen	N-terminal sequence
<i>Lol p Ia</i>	IAKV?PG??I TAEYGDKWLD AKSTWYGKPT
15 <i>Lol p Ib</i>	ADAGYTPAA? ?TPATAP?T
Clone 12R	ADAGYTPAAA ATPATPAATP AAAGGKATTD EQKL
<i>Lol p II</i>	AAPVEFTVEK GSDEKNLALS IKYNKEGDSM A
<i>Lol p III</i>	-TKVDLTVEK GSDAKTLVLN IKYTRPGDTL A
<i>Phl p V</i>	ADLGYPATP AAPGAGYTPA TPAAP
20 <i>Dac g V</i>	GYTPATPAAA GGKATTEEOK L
<i>Poa p IX</i>	ADVGYGAPAT LATPATPAAP AAGYTPAAPA GAAP

Individual protein components were isolated using preparative isoelectric focussing on the Rotofor (Biorad,
25 Richmond, CA). The proteins were separated on SDS-PAGE, and transferred to PVDF membrane (Millipore, Bedford, MA). N-terminal sequencing was performed according to Matsudaira (1987) *J. Biol. Chem.* 262: 10035-10038, and Simpson et al. (1989) *J. Chromatogr.* 476: 345-361.

30 The 31/33 kD protein, *Lol p Ib*, has a different N-terminal amino acid sequence from *Lol p I* (Cottam et al. (1986) *Biochem J.* 234: 305-310; Table 2), described herein as *Lol p Ia*. It is concluded that the allergen encoded by clone 12R represents a major newly identified allergen, *Lol p Ib.1*. The
35 nucleotide sequence of clone 12R is shown in Figures 3b and 3c.

SUBSTITUTE SHEET

- 50 -

Example 3 - Pollen-specific expression of allergens

Poly A+ RNAs were isolated from different plant tissues: seed, leaf, root and pollen. 20 ug of total RNA from the different tissues was electrophoresed on a 1.2% w/v agarose gel in the presence of formamide and formaldehyde (Sambrook, et al., supra), transferred to Hybond-C extra (Amersham, Arlington Heights, IL.) and the filters baked at 80°C for 2 h. The 1.2 kb 12R cDNA was radio-labelled with ³²P and incubated with the NC filter at 65°C in the presence of 50% v/v formamide. The membrane was washed with 2xSSC (0.3 M NaCl, 0.3 M sodium citrate, pH 7.0) containing 0.1% w/v SDS at 65°C. Proteins were isolated from the different tissues (flower, leaf, root and pollen) by grinding in 10 mM PBS containing 1 mM PMSF, and immunoblotted (10 ug protein per lane) with the indicated antibodies. The binding was visualized by using ¹²⁵I-goat anti-mouse Ig (Amersham, Arlington Heights, IL.) for MABs, and polyclonal ¹²⁵I-goat anti-human IgE (Kallestad, Chaska, MN.) followed by autoradiography.

Northern blot analysis of RNA prepared from pollen showed high levels of expression of the cloned allergen gene in pollen but not in any vegetative tissues. A prominent band approximately 1.3 kb long observed in pollen RNA is not detectable in RNA from vegetative tissues (Fig. 2a). Pollen-specific RNA expression corresponded to pollen-specific expression of antigens recognized by MABs 40.1, 12.3 and IgE antibodies (Fig. 2b). Specific binding occurred only when pollen and floral tissues (containing pollen) were used as protein source.

Example 4 - Primary Structure Analysis

The cDNA clone 12R was isolated and subcloned into pGEM-3Z vectors (Promega, Madison, WI) and restriction mapped. Various sized restriction fragments were subcloned into pGEM vectors.

The isolated cDNA clone 12R was also subcloned into pBluescript II vectors (Stratagene, La Jolla, CA) and used to transform XL1 -Blue cells (Stratagene, La Jolla, CA). DNA

SUBSTITUTE SHEET

- 51 -

sequence was determined by double-stranded sequencing carried out by the dideoxy chain termination method (Sanger et al. (1977) *Proc. Natl Acad. Sci. USA* 74: 5463-5468) using T7 DNA polymerase (Pharmacia, Piscataway, NJ). Nested deletions were generated from both the T7 and T3 ends using Exo III and S1 nuclease. Plasmid DNA was prepared using a modified alkaline lysis procedure. Deletion clones were size selected for DNA sequencing by electrophoresis on agarose gels. DNA sequencing was performed using T7 DNA polymerase and dideoxy nucleotide termination reactions. [³⁵S]dATP was used as the label. Sequencing reactions were analyzed on 6% polyacrylamide wedge gels containing 8M urea. Internal sequencing primers were synthesized as necessary. The reading frame was confirmed by sequencing two expression subclones in pGEM vector as detailed in Fig. 4. DNA sequence data were analyzed using the PC GENE System (Intelligenetics, Mountain View, CA).

The nucleotide sequence of the cDNA clone 12R is GC-rich (61% GC, Figs. 3b and 3c). As shown in Figures 3b and 3c, there is an open reading frame of 903 bp starting with an ATG initiation codon at nucleotide 40 and terminating with a TGA codon starting at nucleotide 943. The proposed translation initiation site and its flanking sequences share 89% homology with the consensus plant sequence AACAAATGGC (nucleotides 36-44), and can be considered as in optimum context with the presence of a purine at position -3 (nucleotide 37) from the methionine codon. (Cavener and Ray (1991), *Nucleic Acid Res.*, 19:3185-3192) The open reading frame encodes a protein of predicted Mr 29.8 kD.

The predicted protein sequence, which is rich in alanine (32%), has a putative signal or target peptide sequence of 25 amino acids (amino acids -25 through -1 in Figure 3b). This is indicative of a cleaved protein of predicted Mr 27.3 kD. The N-terminal protein sequence of Lol p Ib is identical to the deduced amino acid sequence of clone 12R immediately after the putative cleavage site of the signal peptide sequence. This confirms that the cDNA-12R encodes a Lol p Ib allergenic protein and that the protein has a signal peptide

SUBSTITUTE SHEET

- 52 -

sequence which is cleaved. The protein encoded by the 12R clone has been designated Lol p Ib.1. The deduced amino acid sequence of Lol p Ib.1 is also shown in Figures 3b and 3c).

The signal sequence has features that are typical of other eukaryotic sequences: a relatively hydrophilic sequence of 5 amino acids at the C-terminus, a relatively hydrophobic sequence extending over most of the signal region which becomes more hydrophilic at the N-terminus (Fig. 3d). The amino acids at the C-terminus include alanine at the cleavage site, an aromatic residue tyrosine at -2, and a helix breaker proline at -6, all of which are common features of the C-terminal region of a signal sequence.

A search for consensus glycosylation sequences (Asn-X-Ser/Thr) in the deduced amino acid sequence detected no such sequences. The absence of an N-linked carbohydrate chain on the allergen was confirmed by the lack of deglycosylation following treatment with the enzymes N-glycanase and endo-F glycosidase. Chemical deglycosylation followed by SDS-PAGE showed no decrease in molecular weight of the protein. The 31/33 kD components remained as a doublet, suggesting that the difference in molecular weight is not due to glycosylation. The deglycosylation treatments did not affect IgE binding to the 31/33 kD components. As compared to Lol p Ia which has 5% carbohydrate, no carbohydrate is present in Lol p Ib.

The amino acid sequence for Lol p Ib and deduced amino acid sequence of Lol p Ib.1 show protein sequence homology with the published amino acid sequences determined for Phl p V (Matthiesen and Lowenstein (1991) *Clin. Exp. Immunol.* 21:297-307) and Dac g V (Walsh et al. (1989) *Int. Arch. Allergy Appl. Immunol.* 91:419-425) from direct protein sequencing and deduced from a Poa p IX cDNA clone (Silvanovich et al. (1991) *J. Biol. Chem.* 266:1204-1210). These sequence homologies are indicated in Table 2.

Example 5 - Delineation of IgE- and Mab-Reacting Epitopes

To localize MAb and IgE determinants, an *E. coli* recombinant expression system was employed (Smith and Johnson

SUBSTITUTE SHEET

- 53 -

(1988) Gene 67: 31-40). Using this system, a number of restriction fragments were subcloned into the expression plasmid pGEX 1-3. The "in frame" sub-cloning of full length cDNA into pGEX, expressed the 61 kD fusion protein recognized
5 by both IgE and MAbs 40.1 and 12.3.

The full length cDNA 12R or two restriction fragments 1H and 2P (shown in Figure 4), were subcloned into plasmid expression vector pGEX. The procedure for inducing fusion proteins and preparation of bacterial lysates have been
10 described earlier (Smith and Johnson, supra). The lysates obtained were subjected to reducing SDS-PAGE, followed by transfer to NC membranes. The blots were probed with IgE antibodies, and MAbs 40.1 and 12.3 as described in relation to Fig. 1b, except that ¹²⁵I-anti-human IgE (Kallestad, Chaska,
15 MN.) was used to detect IgE binding.

Immunoblot analysis showed that most of the fusion protein produced is cleaved by bacterial proteases near its fusion site with glutathione-S transferase, generating break-down products which are recognized by IgE antibodies
20 (Fig. 4). The recombinant fusion protein expressed by fragment 2P (GST-2P), although strongly reactive with both MAbs, was not recognized by IgE antibodies in pooled allergic sera. However, the N-terminally truncated protein produced by fragment 1H (GST-1H) was not recognized by either of the MAbs, but was
25 highly reactive with the IgE antibodies.

In this way, two distinct domains of the allergen molecule have been delineated: the N-terminal containing fragment 2P has recognition sites for MAbs 12.3 and 40.1; and the C-terminal containing fragment 1H which shows strong IgE
30 binding and thus has the allergenic determinant(s). Because the two MAbs have different binding specificities (Fig. 1b), the recognition sites for the two MAbs are likely to be different, although in the same fragment. Fine mapping with smaller fragments is needed to delineate the 12.3 and 40.1 binding
35 sites, but these results are sufficient to show that the IgE determinant is different.

SUBSTITUTE SHEET

- 54 -

Example 6 - Intracellular Targeting Of *Lol p Ib* In Ryegrass Pollen

Mature pollen of *Lolium perenne* was prepared for scanning electron microscopy according to established methods (Staff et al. (1990) *Histochem J.* 22: 276-290). For immunocytochemistry, mature anthers were fixed under anhydrous conditions: 0.1% glutaraldehyde, 1% paraformaldehyde in 2,2-dimethoxypropane at 4°C for 2 h and processed for transmission electron microscopy (Staff et al., *supra*). This method has been developed to reduce diffusion of the allergens from their cellular sites in aqueous media. Blocks were polymerized in LR gold resin with 1% benzil at - 25°C under UV illumination and 80 nm thin sections picked up on gold grids. Immuno-labelling was first with primary antibody, MAb 12.3 (specific for *Lol p Ib*) followed by gold-goat-anti-mouse IgG probe (15 nm particle size). This label was silver-enhanced to 40nm particle size (modified from Danscher & Norgaard (1983) *J. Histochem. Cytochem.* 31:1394-1398). A second labelling was performed on the same sections with a mixture of three MAbs, 3.2, 21.3 and 40.1 followed by gold-goat-anti-mouse IgG probe with 15nm particle size. Antibody specificity and method controls run as described previously (Staff et al., *supra*) showed no gold particles at these sites.

Lol p Ia is located in the cytosol and not in the organelles. These findings were obtained using immuno-gold probes with MAbs specific for *Lol p Ia*. As shown herein, MAb 12.3, which is specific for *Lol p Ib*, binds predominantly to the starch grains (Fig. 5a, b). Grass pollen is filled with starch grains which are 1 x 2.5 μ m in size, and originate in the lumen of amyloplasts.

As shown in Figure 5b, the large gold particles located predominantly over the starch grains (large electron-lucent spaces) show binding of MAb 12.3 to *Lol p Ib*, while smaller particles over the cytosol are typical of binding to *Lol p Ia*. Scale bar is 1 μ m. Figure 5c shows the appearance of fresh, viable pollen after exposure to water for 30s, dark field illumination. Most pollen grains burst, extruding their

SUBSTITUTE SHEET

- 55 -

cytoplasmic contents, including starch grains (white particles) through the germinal pore. Scale bar, 30 μ m.

The localization of *Lol p Ib* in the plastids implies that this protein should be transported from the cytosol to the lumen of the plastids during development. For transport to chloroplasts, the proteins which are synthesized in the cytosol are synthesized as large precursors containing a target peptide sequence that is cleaved after transport into the organelle. These intracellular processing steps, synthesis of *Lol p Ib* first as a pre-allergen in the cytosol and transport to the plastid for post-translational modification, may explain the appearance of the doublet 31/33 kD found by immunoblotting. The unprocessed pre-allergen is 33 kD, and after processing in the plastids, the mature protein is 31 kD. Both these forms co-exist in mature pollen. Alternatively, this doublet may also represent different isoforms or family members of *Lol p Ib*.

Example 7 - Presentation of *Lol p Ib* to the immune system

When the rye-grass flower opens, the anthers are exerted and the pollen is released into the air through a pore which opens at the base of each anther. Rye-grass shows the greatest pollen production of any grass, releasing approximately 460 kg of pollen per hectare into the atmosphere in pastures that are not mowed or grazed. Ninety-nine per cent of this pollen is deposited (and re-deposited) within 1 km of its source. Grass pollen is short-lived, yet it can remain for several days in the atmosphere. Experiments show that the pollen remains viable for only a few hours after release.

When viable, the grains can germinate on the stigma, or in artificial media with high levels of osmoticum. Living viable rye-grass pollen grains when exposed to water, burst at the single germinal aperture releasing the cytoplasmic contents (Fig. 5c). Prominent among the released contents are the starch grains. Media with high osmoticum, e.g. 30% w/v sucrose are required to maintain tonicity of the grains. In contrast, it is well-known that dead pollen grains which have no

SUBSTITUTE SHEET

- 56 -

permeability barriers, act like a sponge. Cellular proteins, including allergens, are released from the surface upon moistening.

It is easy to see how grass pollen can trigger hay fever after contacting the oral and eye mucosa, by direct release of the allergens. The pollen grains themselves remain on the surface of the mucosa, but the released allergenic protein pass through the mucosa and subepithelial layers where they interact with basophils and mast cells. It is less easy to see how pollen grains as large as 30-50um in diameter can induce allergic asthma, a disease triggered by the presence of allergens in the airways of the lungs.

Recent evidence suggests that grass pollen allergens are associated with smaller micronic particles found in the atmospheric aerosol. The original of such particles is obscure. From the present results on allergen localization, and observations on pollen behavior in water, a new hypothesis is proposed to explain how grass pollen can induce allergic asthma in the lungs of susceptible humans. Starch grains are released as micronic particles into the atmospheric aerosol when the living pollen grains encounter water vapor, or water on the surface of a leaf or other substrata. These particles, both coated and filled with allergens, act as vehicles for allergen presentation to the upper and lower respiratory tract. Micronic particles can also, of course, results from the leaching of allergens from grass pollen and deposition on other components of the atmospheric aerosol.

Example 8 - Monoclonal Antibodies Against Lol p Ib.1

Monoclonal antibodies (MAbs) were prepared against fusion protein GST-1H from Example 5 using techniques that are well known to those skilled in the art (see for example, Kohler and Milstein, *supra*, and Kohler and Milstein, *supra*). The fusion protein encoded by fragment 1H (Figure 4), which corresponds to the IgE binding domain, is antigenic.

Four female BALB/c mice were injected intraperitoneally (i.p.) with 100 mg of FPLC purified GST-1H fusion protein in

SUBSTITUTE SHEET

- 57 -

0.1ml PBS and 0.1ml RIBI adjuvant (Immunochem. Res., Hamilton, MT). Fourteen days later a booster i.p. of the same material was given. After ten days the mice were bled. The serum was screened for binding to Western blots of total ryegrass pollen proteins and the mice were selected on the basis of this serum binding to the blot. Fourteen days later the mouse selected for fusion was given an i.p. booster of 0.2 ml containing 100mg fusion protein only. Four days later the mouse was sacrificed and the spleen removed for fusion with myeloma cells (a gift from the Veterinary Research Institute, Parkville, Victoria, Australia). The methods used for fusion and culture were based on those of Harlow and Lane (1990) Antibodies, A Laboratory Manual, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY), using RPMI and Hybridsera (Commonwealth Serum Laboratories, Melbourne, Victoria, Australia). Aminopterin selection was used (50X HAT and HT solutions, Flow Laboratories, Scotland, U.K.) Cloning was by limiting dilution.

Monoclonal lines were isotypized using a mouse monoclonal antibody isotyping kit (Amersham International, U.K.). Human allergic sera were collected, after informed consent, from patients who showed typical symptoms of seasonal hay fever during the grass pollen season and gave a positive response to a skin prick test. The sera were assayed for IgE reactivity with total proteins of ryegrass pollen on Western blots. The pollen samples were purchased from Greer Laboratories (Lenoir, NC). Soluble proteins were extracted from the grass pollen by vigorous shaking in PBS containing 1mM phenyl-methylsulfonyl fluoride on ice for three hours. The protein concentration for each sample was determined using Bio-Rad (Richmond, CA) protein assay.

The antibody binding of each grass was initially detected by slot immunoblotting. 100 µl sample containing 2mg of total pollen proteins was applied to the nitrocellulose membrane using a Minifold II slot blotting apparatus (Schleicher and Schuell, Dassel, Germany). this was washed in PBS and blocked in the same buffer containing 10% milk powder.

SUBSTITUTE SHEET

- 58 -

SDS-PAGE was carried out with a 10-15% acrylamide gradient gel utilizing a Bio-Rad (Richmond, CA) Protean II slab gel apparatus and the Laemmli buffer system (Laemmli, U.K. (1970) *Nature* 227: 680). The proteins were visualized by
5 Coomassie Blue R250 (Sigma Chemical Co., St. Louis, MO) staining. Proteins separated by gradient SDS-PAGE were electrophoretically transferred from the gel onto nitrocellulose membrane according to the procedure of Towbin et al., (1979) *Proc. Natl. Acad. Sci. USA* 76: 4350-4354, in a
10 Bio-Rad (Richmond, CA) transblot cell. The proteins on the nitrocellulose were detected by blocking the non-specific sites by incubating the membrane in milk powder as described for slot blots. The membrane was then washed in PBS and immersed in either the MAb solution for two hours or overnight in human
15 serum (a pool of sera obtained from 10 grass-allergic patients) diluted 1:5 in PBS containing 0.5% BSA and 0.1% sodium azide. The membrane, incubated in the MAb solution, was washed in PBS, then incubated with sheep anti-mouse IgG-horseradish peroxidase (Silenus, Australia) diluted 1:500 in PBS-BSA. After washing,
20 the serum blot was first incubated in a solution of rabbit anti-human IgE (Dakopatts, Glostrup, Denmark) diluted 1:200 in PBS-BSA for two hours and then in a solution of goat anti-rabbit IgG-horseradish peroxidase (Promegal, Madison, WI) diluted 1:2500 with PBS-BSA for one hour. After washing the
25 antibody binding to the blots was visualized by incubating in peroxidase substrate solution containing 4-chloro-1-naphthol and hydrogen peroxide.

The fractions containing the GST-1H fusion protein were fractions 9 and 10 as eluted from the FPLC column. These
30 fractions, when analyzed by SDS-PAGE revealed a single band of 41kD (GST-1H) corresponding to 26kD GST and 15kD protein encoded by fragment 1H.

During MAb production, one fusion resulted in 75 wells containing cell colonies, 7 of which were positive to native
35 Lol p Ib pollen proteins. Three strongly growing colonies were cloned to produce MAb lines. When isotyped two lines produced IgG kappa antibodies, designated LpIX-3A and LpIX-4A, and one

SUBSTITUTE SHEET

- 59 -

produced IgM kappa antibody. In a similar fashion, the MAb LpI-7E (7E) was generated using soluble pollen extract as an antigen. MAb LpI-7E is specific for *Lol p Ia*.

These MABs bind to non-denatured antigens in the pollens of *Dactylis glomerata*, *Festuca elatior*, *Lolium perenne*, *Lolium multiflora* and *Poa pratensis* (Figure 6). On Western blots of soluble pollen proteins separated by SDS-PAGE, MABs LpIX-3A and LpIX-4A bind to antigens in *Festuca elatior*, *Lolium perenne*, *Lolium multiflora* and *Poa pratensis* (Figure 7). These grasses are all taxonomically related. They are members of the tribe Poeae, supertribe Poadae, subfamily Pooideae.

Example 9 - Isolation of cDNA Clone 19R Encoding *Lol p Ib.2* Immunological Screening

Duplicate filters of the cDNA expression library in Example 1 were screened with specific IgE from pooled human allergic sera. The bound IgE was detected using ¹²⁵I-labelled anti-human IgE (Kallestad Laboratories, Chaska, MN). The plaques that were antibody-positive on both of the duplicate filters were picked off, purified, and then replated and tested for binding to MABs.

Plaque purified clone 19R was not positive when tested with non-allergic sera (Fig. 8). This showed that clone 19R is an allergen by its ability to bind specific IgE in sera of ryegrass sensitive patients.

Clone 19R was digested with EcoRI and ligated into pGEM plasmid (Promega, Madison, WI). Fig. 9 shows a partial restriction map of the subcloned EcoRI insert from clone 19R compared to restriction maps of the genes encoding *Lol p Ib.1* and *Lol p Ia*. The insert is different from those encoding *Lol p Ia* and *Lol p Ib.1*, as shown in Fig. 9. The size of the EcoRI insert is about 1295 bp.

Subcloning and Sequencing of DNA

DNA was prepared from plaque-purified phase using the liquid lysate method as described in Meese, E. et al., (1990) *Nucleic Acids Res.*, volume 18:1923. Inserts recovered from EcoRI digestion were ligated into pGEM4 -Z (Promega, Madison,

SUBSTITUTE SHEET

- 60 -

WI) and subcloned as various-sized restriction fragments into pGEM vectors (pGEM4 -Z). All sequencing was done using double stranded plasmid templates. These templates were prepared as described in the Qiagen, Inc., Chatsworth, CA, USA). Dideoxy sequencing (Sanger et al, (1977) *Proc. Nat'l. Acad. Sci. USA*, 74:5460-5463) was performed as described in Example 4. 7-deaza dITP was used to resolve severe GC band compressions. Sequencing was facilitated by generation of nested deletions from both ends of the insert with Exo III and S1 nucleases. Internal sequencing primers were synthesized as necessary.

Sequence Analysis

Sequence analysis was carried out using the Melbourne database system (MELBDBSYS), a collection of analysis programs developed at the Walter and Eliza Hall, Ludwig and Howard Florey Institutes of the University of Melbourne, Australia, and PC Gene (Intelligenetics, Mountain View, CA). This system incorporates databases from the following sources: GenBank, EMBL, and BPRF nucleic acid libraries; NBRF PRI protein, PSD-Kyoto (Ooi), GBtrans, Swiss-Prot, and Doolittle protein libraries. During the searching period, EMBL and GenBank databases were releases 28.0 and 68.0 respectively.

The cDNA sequence of clone 19R is shown in Figures 10a and 10b and contains 1295 nucleotides. There is an open reading frame of 1017 bp starting with an ATG initiation codon at nucleotide positions 25-27 and terminating with a TGA stop codon starting at nucleotide position 1041. The cDNA of clone 19R possesses the following characteristics suggesting that it contains full-length coding regions:

i) The proposed translation initiation site and its flanking sequences (nucleotides 21-29) share 89% homology with the consensus sequence of monocot plants. The most critical nucleotide, a purine at position -3 relative to the ATG start codon (nucleotide 21 in Figure 10a), is conserved, Cavener, D.R., and Ray, S.C. (1991) *Nucleic Acids Research*, 19:3185-3192;

ii) The cDNA has a complete 3'-untranslated region canonical AATTAA polyadenylation signal, Birnsteil et al.

SUBSTITUTE SHEET

- 61 -

(1985) *Cell*, 41: 349-359, followed by a poly (A) tail; and

iii) The 3'-untranslated region also contains ATTTA which may be associated with mRNA stability.

The nucleotide sequence of clone 19R cDNA is G+C rich (63%). The open reading frame potentially encodes a protein, designated Lol p Ib.2 of 314 amino acids with a predicted Mr of 35.3 kD. The predicted protein appears to possess a leader peptide of 25 amino acids on the basis of hydropathy profiles of the N-terminal sequence as shown in Fig. 11, which shows the hydrophobicity profile of the predicted amino acid sequence based on the method of Kyte and Doolittle (1982) *J. Mol. Biol.*, 157:105-132, with a window of nine amino acids. This suggests the molecular weight of the mature processed protein is 32.8kD. No Asn-X-Ser/Thr sequons required for N-glycosylation are found and the predicted pI value for the mature protein is 5.9.

Nucleotide and amino acid sequence searches of existing data bases showed that clone 19R only has similarity with Lol p Ib.1 and Poa p IX allergens. There is a 72.3% homology between the nucleotide coding regions of clone 19R and clone 12R, as shown in Figures 12a and 12b. Amino acid comparison showed 66.8% identity between the predicted amino acid sequences of clone 19R and Lol p Ib.1 as shown in Figure 13. Both allergens have a very similar 25 amino acid leader peptide. There is a 64-69% identity between the amino acid sequences of clone 19R and the three isoallergens of Poa p IX (Silanovich et al. (1991) *J. Biol. Chem.*, 266:1204-1210).

Isolation of Pollen Proteins and Immunoblotting

Soluble proteins were extracted from ryegrass pollen by vigorous shaking in PBS and 1 mM phenylmethylsulfonyl fluoride on ice for three hours. Conditions for SDS-PAGE were essentially as described in Ong et al. (1990) *Int. Arch. Allergy Appl. Immunol.*, 93:338-343. Immediately after electrophoresis, the separated proteins were either silver stained (Angorge, W. (1982), in "Electrophoresis '82: Advanced Methods, Biochemical and Clinical Applications, Proceedings of the International Conference on Electrophoresis, Athens, Greece, April 21-24, 1982", Editor: D. Stathakos, Walter de

SUBSTITUTE SHEET

- 62 -

Gruyeer, Berlin and New York, 1983, pages 235-242) or transferred at 4°C to nitrocellulose (Towbin et al., (1979) *Proc. Natl. Acad. Sci. USA*, 76:4350-4354.

For IgE antibody binding, blots were incubated in
5 pooled allergic sera or affinity purified IgE in PBS containing
0.5% bovine serum albumin (BSA). The bound IgE was detected
according to the method of Ong, E. et al., (1990) *Int. Arch.*
Allergy Appl. Immunol., 93:338-343, using ¹²⁵I-labeled
anti-human IgE (Kallestad Lab, Chaska, MN). For MAb binding,
10 the bound IgG was detected using horseradish peroxidase labeled
sheep anti-mouse Ig (Silenus, Hawthorn, Victoria, Australia).
The blot was developed using an enhanced chemiluminescence
system (Amersham Int., U.K.).

Affinity Purification of IgE Antibodies

15 cDNA clones in lambda gt 11 phages encoding allergens
were expressed as fusion proteins in *Escherichia coli*. Plaque
lifts containing the recombinant fusion proteins (rfp) were
then incubated in pooled sera. The bound IgE antibodies were
eluted with 0.2 M glycine HCL, pH 2.6/0.5% BSA/0.1% sodium
20 azide and used to probe Western blots. Binding of IgE was
visualized using ¹²⁵I-labeled anti-human IgE (Kallestad, Chaska,
MN) followed by autoradiography, Ong et al., *Int. Arch Allergy*
Appl. Immunol., 93:338-343.

RNA Blot Hybridization

25 For RNA gel blot analysis, total RNA was denatured in
20 mM 3-(N-morpholino)-propanesulfonic acid, 50% deionized
formamide, and 2.2 M formaldehyde at 65°C for five minutes,
electrophoresed in 1.2% agarose gel containing 2.2 M
formaldehyde, and electrolotted onto nitrocellulose. The RNA
30 slot-blot analysis was carried out by denaturing the total RNA
is 20 mM 3-(N-morpholino)-propanesulfonic acid, 5mM sodium
acetate, and 1mM EDTA at 65°C for ten minutes and applying the
samples onto nitrocellulose saturated with 20 xSSC (SSC - 3 M
sodium chloride, 1.0 M sodium citrate) fitted in the Minifold
35 11 Filtration Manifold (Schleicher & Schuell, Dassel, Germany).
Both filters were prehybridized for two to six hours at 42°C in
a solution containing 50% deionized formamide, 2 x SSPE (SSPE

SUBSTITUTE SHEET

- 63 -

- 3 M sodium chloride, 0.2 M sodium phosphate, 0.02 M EDTA), 1% sodium dodecyl sulfate (SDS), 0.5% Blotto (10% non-fat milk in phosphate buffered saline), 10% dextran sulfate, and 0.5 mg/ml ³²P-labelled cDNA probe prepared by random oligonucleotide priming using an oligolabelling kit (Bresatec, Adelaide, Australia). The filters were washed in four changes of 2 x SSC, 0.1% SDS at 42°C for 2 hours, and then exposed to X-ray film.

To determine the tissue specificity of clone 19R gene expression, Northern blot analysis of RNA prepared from various *L. perenne* tissues were examined. The Northern blot was probed with an 84 bp Ssp I/Eco RI restriction fragment from clone 19R. This cDNA probe, corresponding to nucleotides 1207 to 1291 of clone 19R (Figure 10b) that binds to clone 19R but not clone 12R, hybridizes to a single transcript of 1780 bases in pollen. No hybridization to any transcripts in ryegrass seed, root and leaf was observed. A positive control hybridization using a complete ribosomal DNA from *Pisum sativum* as the probe, showed that the amount of RNA used was sufficient for detection in all the samples. This is shown in Figures 14a and 14b.

Example 10 - Characterization of *Lol p Ib* Allergens Material and Methods

Pollen was obtained from Greer Laboratories, Lenoir, NC. Soluble pollen proteins were extracted as described in Griffith, et al., (1991) *FEBS Letters*, 279:210-215. Crude pollen extract was obtained and its protein concentration determined as described in Ong, et al., (1990) *Int. Arch. Allergy Appl. Immunol.*, 93:338-343. MAbs LpIX3A and LpIX4A were raised against a recombinant protein encoded by the IgE binding portion of clone 12R, as described in Example 8. MAb 7E is specific for *Lol p Ia* as described in Example 8 and FMC-A1 is as described in Example 1.

Serum used in the experiment to identify allergen regions of *Lol p Ia* and *Lol p Ib* was collected by Dr. R. Phomley at the Epworth Hospital (Richmond, Australia) from patients with a history of allergy to ryegrass pollen and a

SUBSTITUTE SHEET

- 64 -

positive skin test to ryegrass pollen extract. IgE was affinity-purified from recombinant allergens as previously described (Singh, et al., (1991) *Proc. Nat'l Acad. Sci. USA*, 21:309; Example 2) except recombinant proteins were derived from lambda-gt11 culture rather than pGEX cultures.

Sera used in the experiment identifying fragments of clone 12R and clone 19R which encode IgE binding polypeptides were obtained from fifty subjects selected on the basis of previous clinical record of spring hay fever symptoms and RAST towards ryegrass pollen (Phadezyme RAST, Pharmacia LKB, Sweden). The RAST score for all sera was four. Sera were also obtained from two subjects, who were shown to be non-atopic by RAST, and used as negative controls. Sera were stored at -20°C in small aliquots.

15 Two Dimensional Gel Electrophoresis and Immunoblot Analysis

Two dimensional (2D)-PAGE was performed in a mini-Protean II 2-D cell (Biorad, Richmond, CA) according to the manufacturer instructions. The proteins were diluted 1:1 in 4% CHAPS. An aliquot of 13mg of protein was applied per gel and the sample overlaid with first dimension sample overlay buffer. The first dimension gel was run for 3.5 hours. The second dimension gel was run for 45 minutes. Proteins on 2D-PAGE gels were silver stained to reveal the protein profile.

Conditions for electrophoresis and Western blotting and for the processing of Western blots with MAbs and IgE were as described by Singh, et al., (1985) *Int. Arch. Allergy Appl. Immun.*, 78:300.

Identification of allergenic isoforms in pollen extract.

Probing of Western blots of pollen proteins separated by SDS-PAGE with sera of allergic individuals reveals protein bands at four different molecular weights in the MW range of 28-35 kD that bind IgE. Similar treatment of Western blots of 2D-gels resolved these four bands into twelve allergen spots, as shown in Fig. 15. Using a number of MAbs and IgE preparations the antigenic relationship between these allergens was studied.

SUBSTITUTE SHEET

- 65 -

Two-dimensional Western analyses are shown in Fig. 16 and Table 3. Blots were probed with affinity purified IgE antibodies from *Lol p Ib.1*, *Lol p Ib.2*, rfp *Lol p Ia*, total pooled sera and MAb FMC A7. The total pooled sera have IgE antibodies recognizing two acidic isoforms of the 32 kD component (no. 1, 2), five isoforms of the 30 kD component with pI values in the range of 5-11 (bands 3-7) and a basic band (no. 8) of 28 kD molecule (Fig. 16, panel b). The affinity-purified IgE antibodies from *Lol p Ib.1* and *Lol p Ib.2* bound to all the isoforms of 28/30/32 kD molecules except isoform no. 5 (Fig. 16, panels d and e). In contrast, MAb FMC-A7 recognized the 32 kD isoforms (bands 1 and 2), two acidic isoforms (bands 3,4) and a basic band no. 7 of the 30 kD component (Fig. 16, panel c).

15 The relative allergenicity of *Lol p Ia*, *Lol p Ib.1* and *Lol p Ib.2* were tested using 30 individual allergic sera. Fig. 17 shows that 27 of the patients (90%) have IgE antibodies reactive to *Lol p Ia* and 6 of them (20% (Fig. 17) have IgE antibodies specific to *Lol p Ia* (did not bind to either *Lol p Ib* isoform). Twenty-four patients (80%) have IgE antibodies recognizing both *Lol p Ib.1* and *Lol p Ib.2* recombinant isoforms. There were 3 patients (10%) who possessed IgE antibodies only recognizing the *Lol p Ib* isoforms (did not bind to *Lol p Ia*).

SUBSTITUTE SHEET

- 66 -

Table 3

Characteristics of Allergens on Two Dimensional Gels

	Allergen No.	MW (kD)	pI	Mab binding ^a	IgE binding ^b	Group
5	1	34	5.5	A1, 7E	AP13R TIgE	Ia
	2	34	5.9	A1, 7E	AP13R TIgE	Ia
	3	34	6.45	A1, 7E	AP13R TIgE	Ia
	4	34	7	A1, 7E	AP13R TIgE	Ia
10	5	32	6	A1, 3A, 4A	AP12R, AP19R TIgE	Ib
	6	32	6.45	A1, 3A, 4A	AP12R, AP19R TIgE	Ib
	7	30	6	A1, 3A, 4A	AP12R, AP19R TIgE	Ib
15	8	30	6.45	A1, 3A, 4A	AP12R, AP19R TIgE	Ib
	9	30	7.2	A1, 3A	AP12R, AP19R TIgE	Ib
20	10	30	8.3	A1, 3A	AP12R, AP19R TIgE	Ib
	11	30	10.6	A1, 3A	AP12R, AP19R TIgE	Ib
	12	28	8.0	A1, 3A	AP12R, AP19R TIgE	Ib
25	13	60	7		TIgE	IV
	14	60	7.3		TIgE	IV
	15	60	9		TIgE	IV
	16	14	<5.65		TIgE	II
	17	14	<5.65		TIgE	II
30	^a A1- antibody FMC-A1; 3A- antibody LpIX3A; 4A- antibody LpIX4A; 7E-LpI-7E.					
35	^b AP12R - IgE affinity purified from recombinant <i>Lol p</i> Ib.1, fragment IH. AP13R - IgE purified from recombinant <i>Lol p</i> Ia clone 13R. AP19R-IgE affinity purified from recombinant <i>Lol p</i> Ib.2.					

TIgE - IgE from whole serum of allergic individuals.

Example 11 - Extraction of RNA From *Lolium perenne* Flowerhead And Polymorphism Analysis Of The Genes Encoding *Lol p* Ib.1 and *Lol p* Ib.2 By Polymerase Chain Reaction (PCR)

40 Fresh flowerheads were collected from *Lolium perenne* grass in Australia, frozen and shipped to the United States.

SUBSTITUTE SHEET

- 67 -

500 mg of flowerhead was ground by mortar and pestle on dry ice and suspended in 5 ml of 50 mM Tris pH 9.0 with 0.2 M NaCl, 1 mM EDTA, 0.1% SDS that had been treated overnight with 0.1% DEPC, as described in by Frankis and Mascarhenas (1980) Ann. 5 45: 595-599. After one extraction with phenol/chloroform/isoamyl alcohol (mixed 25:24:1), the material was sonicated in the phenol/chloroform/isoamyl alcohol for 60 seconds and re-extracted. The sonication was repeated at the third extraction, for 30 seconds. Two final extractions were 10 performed without sonication. The RNA was precipitated from the aqueous phase with 0.1 volume 2M sodium acetate and 2 volumes ethanol. The pellets were recovered by centrifugation, resuspended in dH₂O and heated to 65°C for 5 minutes. Two ml of 4M lithium chloride was added to the RNA preparation and 15 precipitated overnight at 0°C. The RNA pellets were recovered by centrifugation, resuspended in 1 ml dH₂O, and again precipitated with 3M sodium acetate and ethanol on dry ice for one hour. The final pellet was washed with 70% ethanol, air dried and resuspended in 100 µl DEPC-treated H₂O and stored at 20 -80°C.

First strand cDNA and double stranded cDNA were each synthesized from 7.5 µg flowerhead RNA using a commercially available kit (cDNA synthesis system plus kit, BRL, Gaithersburg, MD). The second strand cDNA reaction mixture was 25 phenol extracted, ethanol precipitated, and blunted with T4 DNA polymerase (Promega, Madison, WI). This double stranded cDNA was ligated to ethanol precipitated, self annealed, oligonucleotides AT and AL for use in a modified Anchored PCR reaction, according to the method of Rafner et al. (1990) J. 30 Biol. Chem. 266: 1229-1236 ; Frohman et al. (1990) Proc. Natl. Acad. Sci. USA 85: 8998-9002; and Roux et al. (1990) BioTech. 8: 48-57. Oligonucleotide AT has the sequence 5'-GGGTCTAGAGGTACCGTCCGATCGATCATT-3' (Rafner et al. supra). Oligonucleotide AL has the sequence 5'-AATGATCGATGCT-3' (Rafner 35 et al. supra.)

The amino termini of clone 12R and clone 19R were amplified from the linkered cDNA (2 µl reaction). PCR were

SUBSTITUTE SHEET

- 68 -

carried out using a commercially available kit (GeneAmp DNA Amplification kit, Perkin Elmer Cetus, Norwalk, CT) whereby 10 μ l 10x buffer containing dNTPs was mixed with 100 pmol each of the oligonucleotide AP-2 and LP5-8 primers (ED:EDT in a 3:1 M ratio), cDNA (2 μ l of the linkered cDNA reaction mix), 0.5 μ l Amplitaq DNA polymerase, and distilled water to 100 μ l. The samples were amplified with a programmable thermal controller (MJ Research, Cambridge, MA). The temperature cycling program used was as follows: denature template DNA, 94°C, 1 min.; anneal oligonucleotides, 65°C, 1 min. 30 sec.; elongate, 72°C, 2 min.; repeat for 24 cycles; hold at 4°C.

LP5-8 has the sequence 5'-GCCTTGAAGCC(A/G)GCGTTGA-3' wherein position 12 is either an A or a G. LP5-8 corresponds to the non-coding strand sequence complementary to nucleotides 227 to 244 of *Lol p Ib.1* (Figures 3b and 3c) and nucleotides 248 to 265 of *Lol p Ib.2* (Figures 10a and 10b). AP-2 has the sequence 5'-GGGTCTAGAGGTACCGTCC-3'. The primary reaction was carried out as described herein. Two percent (2 μ l) of this initial amplification was then used in a secondary amplification with 100 pmol each AP-2 and LP5-9, an internally nested *Lol p Ib.1/Ib.2* oligonucleotide primer. LP5-9 has the sequence 5'-TTGGATCCTCGGTCGTCGCCCTTCCCT-3' wherein the nucleotides 5'-TTGGATCC-3' (bases 1 through 8 of LP5-9) were added to create a Bam HI restriction site and nucleotides 9-26 correspond to non-coding strand sequence complementary to nucleotides 186 to 203 of *Lol p Ib.1* (Figures 3b and 3c) and nucleotides 207 to 224 of *Lol p Ib.2* (Figures 10a and 10b). The dominant amplification product was a DNA smear from about 100 - 250 base pairs on a ethidium bromide (EtBr) - stained 3% GTG agarose gel.

Amplified DNA was recovered by sequential chloroform, phenol, and chloroform extraction, followed by precipitation on dry ice with 0.5 volumes of 7.5M ammonium acetate and 1.5 volumes of isopropanol. After precipitation and washing with 70% ethanol, the DNA was simultaneously digested with Xba I and Bam HI in a 50 μ l reaction, precipitated to reduce the volume to 20 μ l and electrophoresed through a preparative 2% GTG

SUBSTITUTE SHEET

- 69 -

NuSeive low melt gel (FMC, Rockport, ME). The appropriate sized DNA was visualized by EtBr staining, excised, and ligated into appropriately digested pUC19 for sequencing by the dideoxy chain termination method of Sanger et al. (1977) *Proc. Natl. Acad. Sci. USA* 74: 5463-5476) using commercially available sequencing kits (Sequenase kit, Taqence kit, both from U.S. Biochemicals, Cleveland, OH).

All clones were derived from a single PCR reaction and amino acid differences only represent potential polymorphisms. These amino acid differences need to be confirmed in an independent PCR due to the inherent error rate in Taq polymerase (Saiki et al. (1988) *Science* 239:487-491).

Eleven clones were found to contain sequences homologous to clone 12R. Two clones were found to be homologous to clone 19R. Potential nucleotide polymorphisms in clone 12R resulting in amino acid differences in *Lol p Ib.1* are shown in Table 6.

Table 6

	Amino Acid Position	Amino Acid Change
20	-8	A→V
	8	A→P
	15	T→N
	35	L→R
25	36	E→T
	44	A→S
	50	A→P

A potential polymorphism in clone 19R resulting in an amino acid change in *Lol p Ib.2* was found in one clone where the amino acid at position 30 was changed from T→S.

The cDNA encoding an internal portion of clone 12R was cloned from first strand cDNA using oligonucleotides LP5-5 and LP5-6 in a PCR reaction using the above described temperature cycling program. LP5-5 has the sequence 5'-GGGAATTCACCGACGAGCAGAAGCTG-3' wherein bases 1 through 8 (5'-GGGAATTC-3') of LP5-5 were added to create an Eco RI restriction site for cloning purposes and bases 9 to 26 corresponded to nucleotides 199 to 216 of clone 12R and nucleotides 220 to 237 of clone 19R. LP5-6 has the sequence

SUBSTITUTE SHEET

- 70 -

5'-GGGGATCCCTGGGTCATGGCGGTGAT-3' wherein bases 1 through 7 (5'-GGGGATC-3') (bases 1 through 7 of LP5-6) were added to create a Bam HI restriction site for cloning purposes and bases 8 to 26 were complementary to nucleotides 808 to 826 of clone 12R. The dominant amplified product was a DNA band of approximately 620 base pairs. The amplified DNA product was purified and precipitated as above, followed by digestion with Eco RI and Bam HI and electrophoresed through a preparative 2% low melt gel. The dominant DNA band was excised and ligated into appropriately digested pUC19 for sequencing. Several clones were obtained containing the clone 12R internal sequence.

All clones were derived from a single PCR reaction and amino acid differences only represent potential polymorphisms. These amino acid differences need to be confirmed in an independent PCR due to the inherent error rate in Taq polymerase (Saiki et al. (1988) Science 239:487-491.

Potential polymorphisms of the Lol p Ib.1 internal sequence are shown in Table 7.

Table 7

	Amino Acid Position	Amino Acid Change
	72	L→F
	76	S→P
25	78	A→T
	80	A→R or G
	84	I→V
	93	V→I or R
	147	L→H
30	148	Q→A
	149	I→V
	169	T→A
	170	N→D
	179	A→S
35	185	N→K
	207	Q→A
	214	A→P
	232	I→M

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all of the steps,

SUBSTITUTE SHEET

- 71 -

features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.

5

SUBSTITUTE SHEET

- 72 -

Claims

1. A purified nucleic acid sequence coding for at least one *Lol p Ib* ryegrass pollen allergen, or at least one antigenic fragment thereof, or derivative or homologue thereof, or the functional equivalent of said nucleic acid sequence.
2. A nucleic acid sequence of claim 1 wherein said nucleic acid sequence consists essentially of a nucleic acid sequence encoding amino acids -25 through 276 of *Lol p Ib.1* as shown in Figures 3b and 3c.
3. A nucleic acid sequence of claim 1 wherein said nucleic acid sequence consists essentially of at least one fragment of the coding portion of the nucleic acid sequence encoding *Lol p Ib.1* shown in Figures 3b and 3c, or the functional equivalent of said nucleic acid sequence.
4. A nucleic acid sequence of claim 1 wherein said nucleic acid sequence consists essentially of a nucleic acid sequence encoding amino acids -25 through 314 of *Lol p Ib.2* shown in Figures 10a and 10b.
5. A nucleic acid sequence of claim 1 wherein said nucleic acid sequence consists essentially of at least one fragment of the coding portion of the nucleic acid sequence encoding *Lol p Ib.2* shown in Figures 10a and 10b, or the functional equivalent of said nucleic sequence.
6. A nucleic acid sequence of claim 1 wherein said nucleic acid sequence consists essentially of a nucleic acid sequence encoding amino acids 1-276 of *Lol p Ib.1* shown in Figures 3b and 3c.
7. A nucleic acid sequence of claim 1 wherein said nucleic acid sequence consists essentially of a nucleic acid sequence encoding amino acids 1-314 of *Lol p Ib.2* shown in Figures 10a and 10b.
8. An expression vector comprising a nucleic acid sequence coding for at least one *Lol p Ib* ryegrass pollen allergen, or at least one antigenic fragment thereof, or derivative or homologue thereof, or the functional equivalent of said nucleic acid sequence.

SUBSTITUTE SHEET

- 73 -

9. An expression vector of claim 8 wherein said nucleic acid sequence consists essentially of the nucleotide sequence of the coding portion of the nucleic acid sequence for *Lol p Ib.1* shown in Figures 3b and 3c.

5 10. An expression vector of claim 8 wherein said nucleic acid sequence consists essentially of at least one fragment of the coding portion of the nucleic acid sequence encoding *Lol p Ib.1* shown in Figures 3b and 3c.

11. An expression vector of claim 8 wherein said
10 nucleic acid sequence consists essentially of the nucleotide sequence of the coding portion of the nucleic acid sequence encoding *Lol p Ib.2* shown in figures 10a and 10b.

12. An expression vector of claim 8 wherein said
15 nucleic acid sequence consists essentially of at least one fragment of the coding portion of the nucleic acid sequence encoding *Lol p Ib.2* shown in Figures 10a and 10b.

13. An expression vector of claim 8 wherein said
20 nucleic acid sequence consists essentially of a nucleic acid sequence encoding amino acids 1 through 276 of *Lol p Ib.1* shown in Figures 3b and 3c.

14. An expression vector of claim 8 wherein said
nucleic acid sequence consists essentially of a nucleic acid sequence encoding amino acids 1-314 of *Lol p Ib.2* shown in Figures 10a and 10b.

25 15. A host cell transformed to express a protein or peptide encoded by the nucleic acid sequence of claim 1.

16. A host cell transformed to express a protein encoded by the nucleic acid sequence of claim 2.

17. A host cell transformed to express a peptide
30 encoded by the nucleic acid sequence of claim 3.

18. A host cell transformed to express a protein encoded by the nucleic acid sequence of claim 4.

19. A host cell transformed to express a peptide encoded by the nucleic acid sequence of claim 5.

35 20. A host cell transformed to express a protein encoded by the nucleic acid sequence of claim 6.

SUBSTITUTE SHEET

- 74 -

21. A host cell transformed to express a protein encoded by the nucleic acid sequence of claim 7.

22. At least one purified ryegrass pollen allergen *Lol p Ib* family member or at least one antigenic fragment thereof, or derivative or homologue thereof produced in a host cell transformed with the nucleic acid sequence of claim 1.

23. Purified ryegrass pollen allergen *Lol p Ib.1* produced in a host cell transformed with the nucleic acid sequence of claim 2.

24. At least one fragment of purified ryegrass pollen allergen *Lol p Ib.1* or derivative or homologue thereof produced in a host cell transformed with the nucleic acid sequence of claim 3.

25. Purified ryegrass pollen allergen *Lol p Ib.2* produced in a host cell transformed with the nucleic acid sequence of claim 4.

26. At least one fragment of purified ryegrass pollen allergen *Lol p Ib.2* or derivative homologue thereof produced in a host cell transformed with the nucleic acid sequence of claim 5.

27. Purified ryegrass pollen allergen *Lol p Ib.1* produced in a host cell transformed with the nucleic acid sequence of claim 6.

28. Purified ryegrass pollen allergen *Lol p Ib.2* produced in a host cell transformed with the nucleic acid sequence of claim 7.

29. A method of producing at least one *Lol p Ib* ryegrass pollen allergen, or at least one fragment thereof, or derivative or homologue thereof comprising, culturing a host cell transformed with a DNA sequence encoding said at least one *Lol p Ib* ryegrass pollen allergen, or fragment thereof, or derivative or homologue thereof in an appropriate medium to produce a mixture of cells and medium containing said at least one *Lol p Ib* ryegrass pollen allergen, or at least one fragment thereof, or derivative or homologue thereof; and optionally purifying said mixture to produce purified *Lol p Ib* ryegrass pollen allergen, or at least one fragment thereof, or

SUBSTITUTE SHEET

- 75 -

derivative or homologue thereof.

30. The method of claim 29 wherein said at least one *Lol p Ib* ryegrass pollen allergen is *Lol p Ib.1* or *Lol p Ib.2*.

31. A protein preparation comprising at least one *Lol p Ib* ryegrass pollen allergen, or at least one fragment thereof, or derivative or homologue thereof synthesized in a host cell transformed with a DNA sequence encoding said at least one *Lol p Ib* ryegrass pollen allergen, or at least one fragment thereof, or derivative or homologue thereof.

32. A protein preparation of claim 31 wherein said at least one *Lol p Ib* ryegrass pollen allergen is *Lol p Ib.1* or *Lol p Ib.2*.

33. The protein preparation of claim 31 wherein said at least one fragment of said *Lol p Ib* ryegrass pollen allergen is an antigenic fragment.

34. A protein preparation comprising at least one chemically synthesized *Lol p Ib* ryegrass pollen allergen or at least one fragment thereof, or derivative or homologue thereof.

35. The protein preparation of claim 31 wherein said *Lol p Ib* ryegrass pollen allergen is *Lol p Ib.1* having the amino acid sequence as shown in Figures 3b and 3c.

36. The protein preparation of claim 34 wherein said *Lol p Ib* ryegrass pollen allergen is *Lol p Ib.1* having the amino acid sequence as shown in Figures 3b and 3c.

37. The protein preparation of claim 31 wherein said *Lol p Ib* ryegrass pollen allergen is *Lol p Ib.2* having the amino acid sequence shown in Figures 10a and 10b.

38. The protein preparation of claim 34 wherein said *Lol p Ib* ryegrass pollen allergen is *Lol p Ib.2* having the amino acid sequence shown in Figures 10a and 10b.

39. An isolated antigenic fragment of a *Lol p Ib* ryegrass pollen allergen.

40. The antigenic fragment of claim 39 wherein said allergen from ryegrass pollen is *Lol p Ib.1*.

41. The antigenic fragment of claim 39 wherein said allergen from ryegrass pollen is *Lol p Ib.2*

SUBSTITUTE SHEET

- 76 -

42. The antigenic fragment of claim 39 wherein said antigenic fragment comprises at least one T cell epitope of said allergen.

43. The antigenic fragment of claim 40 wherein said
5 antigenic fragment comprises at least one T-cell epitope of Lol
p Ib.1.

44. The antigenic fragment of claim 41 wherein said antigenic fragment comprises at least one T cell epitope of Lol
p Ib.2.

10 45. The antigenic fragment of claim 42 wherein said antigenic fragment further has minimal immunoglobulin E stimulating activity.

46. The antigenic fragment of claim 42 wherein said antigenic fragment does not bind immunoglobulin E specific for
15 ryegrass pollen allergen.

47. The antigenic fragment of claim 43 wherein said antigenic fragment does not bind immunoglobulin E specific for ryegrass pollen allergen or if binding of the fragment to said immunoglobulin E occurs, such binding does not result in
20 histamine release from mast cells or basophils.

48. The antigenic fragment of claim 43 wherein said antigenic fragment does not bind immunoglobulin E specific for ryegrass pollen allergen.

49. The antigenic fragment of claim 44 wherein said
25 antigenic fragment further has minimal immunoglobulin E stimulating activity.

50. The antigenic fragment of claim 44 wherein said antigenic fragment does not bind immunoglobulin E specific for ryegrass pollen allergen.

30 51. The antigenic fragment of claim 39 wherein said antigenic fragment binds immunoglobulin E to a substantially lesser extent than purified native ryegrass pollen allergen binds said immunoglobulin E.

52. The antigenic fragment of claim 39 wherein said
35 antigenic fragment is capable of modifying, in a ryegrass pollen-sensitive individual to which it is administered, the allergic response to ryegrass pollen.

SUBSTITUTE SHEET

- 77 -

53. The antigenic fragment of claim 40 wherein said antigenic fragment is capable of modifying, in a ryegrass pollen-sensitive individual to which it is administered, the allergic response to ryegrass pollen.

5 54. The antigenic fragment of claim 41 wherein said antigenic fragment is capable of modifying, in a ryegrass pollen-sensitive individual to which it is administered, the allergic response to ryegrass pollen.

55. The antigenic fragment of claim 52 wherein said
10 antigenic fragment is capable of modifying B-cell response of the individual to a ryegrass pollen allergen, T-cell response of the individual to a ryegrass pollen antigen, or both the B cell response and the T cell response to ryegrass pollen allergen.

15 56. The antigenic fragment of claim 53 wherein said antigenic fragment is capable of modifying B-cell response of the individual to a ryegrass pollen allergen, T-cell response of the individual to a ryegrass pollen antigen, or both.

57. The antigenic fragment of claim 54 wherein said
20 antigenic fragment is capable of modifying B-cell response of the individual to a ryegrass pollen allergen, T-cell response of the individual to a ryegrass pollen allergen, or both.

58. A nucleic acid sequence coding for the isolated antigenic fragment of ryegrass pollen allergen of claim 40.

25 59. A modified ryegrass pollen protein allergen which, when administered to a ryegrass pollen-sensitive individual, reduces the allergic response of the individual to ryegrass pollen.

60. The modified ryegrass pollen protein allergen of
30 claim 59 wherein said modified ryegrass pollen protein allergen is a modified Lol p Ib.1 protein or derivative or homologue thereof.

61. The modified ryegrass pollen protein allergen of
claim 60 wherein said modified ryegrass pollen protein allergen
35 is a modified Lol p Ib.2 protein or derivative or homologue thereof.

SUBSTITUTE SHEET

- 78 -

62. At least one modified fragment of a Lol p Ib ryegrass pollen allergen which, when administered to a ryegrass pollen-sensitive individual, reduces the allergic response of the individual to ryegrass pollen.

5 63. At least one modified fragment of claim 62 wherein said modified fragment or fragments is at least one modified fragment of Lol p Ib.1 protein or derivative or homologue thereof.

10 64. At least one modified fragment of claim 62 wherein said modified fragment or fragments is at least one modified fragment of Lol p Ib.2 protein or derivative or homologue thereof.

15 65. An isolated protein allergen or antigenic fragment thereof that is immunologically related to Lol p Ib.1 or Lol p Ib.2, or fragment or derivative or homologue of Lol p Ib.2 or Lol p Ib.2.

20 66. The isolated protein allergen or fragment thereof of claim 65 wherein said protein allergen or antigenic fragment thereof is immunologically cross-reactive with antibodies specific for Lol p Ib.1 or Lol p Ib.2, or fragment or derivative or homologue of Lol p Ib.1 or Lol p Ib.2.

25 67. A pharmaceutical composition comprising purified ryegrass pollen allergen Lol p Ib.1 or Lol p Ib.2, or at least one fragment or derivative or homologue of Lol p Ib.1 or Lol p Ib.2 and a pharmaceutically acceptable carrier or diluent.

 68. The pharmaceutical composition of claim 67 wherein Lol p Ib.1 has the sequence of amino acids 1-276 shown in Figures 3b and 3c.

30 69. The pharmaceutical composition of claim 67 wherein Lol p Ib.2 has the sequence of amino acids 1-314 shown in Figures 10a and 10b.

35 70. A method of treating sensitivity to ryegrass pollen allergen in a mammal sensitive to such pollen allergen or an allergen immunologically cross-reactive with ryegrass pollen allergen, comprising administering to said mammal a therapeutically effective amount of the protein preparation of

SUBSTITUTE SHEET

- 79 -

claim 31.

71. A method of treating sensitivity to ryegrass pollen in a mammal sensitive to such pollen or an allergen immunologically cross-reactive with ryegrass pollen allergen, comprising administering to said mammal a therapeutically effective amount of the protein preparation of claim 34.

72. A method of treating sensitivity to ryegrass pollen in a mammal sensitive to such pollen or an allergen immunologically cross-reactive with ryegrass pollen allergen, comprising administering to said mammal a therapeutically effective amount of the protein preparation of claim 32.

73. A method of treating sensitivity to ryegrass pollen in a mammal sensitive to such pollen or an allergen immunologically cross-reactive with ryegrass pollen allergen, comprising administering to said mammal a therapeutically effective amount of the therapeutic composition of claim 68.

74. A method of detecting in a mammal sensitivity to a Lol p Ib ryegrass pollen allergen comprising combining a blood sample obtained from said mammal with an isolated ryegrass pollen protein allergen or antigenic fragment thereof produced in a host cell transformed with the nucleic acid sequence of claim 1 or fragment thereof or chemically synthesized, or a derivative or homologue of said ryegrass pollen protein allergen under conditions appropriate for binding of blood components with the protein or fragment thereof, or derivative or homologue thereof and determining the extent to which such binding occurs.

75. The method of claim 74 wherein the extent to which binding occurs is determined by assessing T cell function; T cell proliferation; B cell function; binding of the protein, or fragment thereof, or derivative or homologue thereof to antibodies present in the blood; or a combination thereof.

76. A method of detecting sensitivity of a mammal to ryegrass pollen allergen comprising administering to said mammal a sufficient quantity of at least one ryegrass pollen allergen Lol p Ib family member or at least one antigenic fragment thereof, or derivative or homologue thereof to provoke

SUBSTITUTE SHEET

- 80 -

an allergic response in said mammal and determining the occurrence of an allergic response in the individual to said ryegrass pollen allergen.

77. Native purified ryegrass pollen allergen *Lol p Ib*
5 or at least one antigenic fragment or derivative or homologue thereof.

78. A recombinant DNA molecule comprising a
eukaryotic or prokaryotic origin of replication, a detectable
marker, a DNA sequence encoding the *Lol p Ib.1* or *Lol p Ib.2*
10 allergenic protein, or at least one fragment thereof, or a
derivative or a homologue thereof, or an allergenic protein
cross-reactive with an antibody to said *Lol p Ib.1* or *Lol p*
Ib.2 protein or its derivatives or homologues and optionally a
promoter sequence capable of directing transcription of said
15 DNA sequence.

79. The recombinant DNA molecule of claim 79 wherein
the promoter is the *Lol p Ib.1* gene promoter or the *Lol p Ib.2*
gene promoter.

80. A method of producing recombinant *Lol p Ib.1*, *Lol*
20 *p Ib.2*, or at least one fragment of *Lol p Ib.1* or *Lol p Ib.2*,
or a derivative or homologue thereof, or an allergenic protein
immunologically reactive to antibodies to *Lol p Ib.1* or *Lol p*
Ib.2 or at least one fragment of *Lol p Ib.1* or *Lol p Ib.2* or
derivative or homologue thereof, comprising culturing an
25 organism containing a replicable recombinant DNA molecule, said
molecule comprising a promoter capable of expression in said
organism, the gene encoding *Lol p Ib.1*, *Lol p Ib.2*, or at least
one fragment thereof, or derivative of or homologue thereof, or
an immunologically related protein of *Lol p Ib.1* or *Lol p Ib.2*
30 located downstream of and transcribed from said promoter, a
selectable marker and a DNA vehicle containing a prokaryotic or
eukaryotic origin of replication, under conditions and for a
time sufficient for said recombinant DNA molecule to be stably
maintained and direct the synthesis of *Lol p Ib.1*, *Lol p Ib.2*
35 or at least one fragment of *Lol p Ib.1* or *Lol p Ib.2*, or
derivative or homologue thereof, or immunological relative
thereof, and optionally isolating the same.

SUBSTITUTE SHEET

- 81 -

81. The method of claim 80 wherein the promoter is the Lol p Ib.1 promoter or homologue or degenerate from thereof and the host organism is one in which said promoter will function.

82. The method of claim 80 wherein the promoter is the Lol p Ib.2 promoter or homologue or degenerate from thereof and the host organism is one in which said promoter will function.

83. A recombinant DNA molecule comprising a ryegrass pollen promoter sequence or homologue or degenerate form thereof located on said molecule and further having one or more restriction endonuclease sites downstream of said promoter such that a nucleic acid sequence coding for the ryegrass pollen allergen Lol p Ib.1 or Lol p Ib.2, or at least one antigenic fragment of Lol p Ib.1 or Lol p Ib.2, or their derivatives or homologues inserted into one or more of these sites is transcribable in the correct reading frame.

84. A monoclonal antibody specific for Lol p Ib.1 wherein said antibody is raised against Lol p Ib.1 of claim 23.

85. A monoclonal antibody specific for Lol p Ib.2 wherein said antibody is raised against Lol p Ib.1 of claim 25.

86. The monoclonal antibody of claim 84 wherein said antibody is cross-reactive between Lol p Ib.1 and Lol p Ib.2.

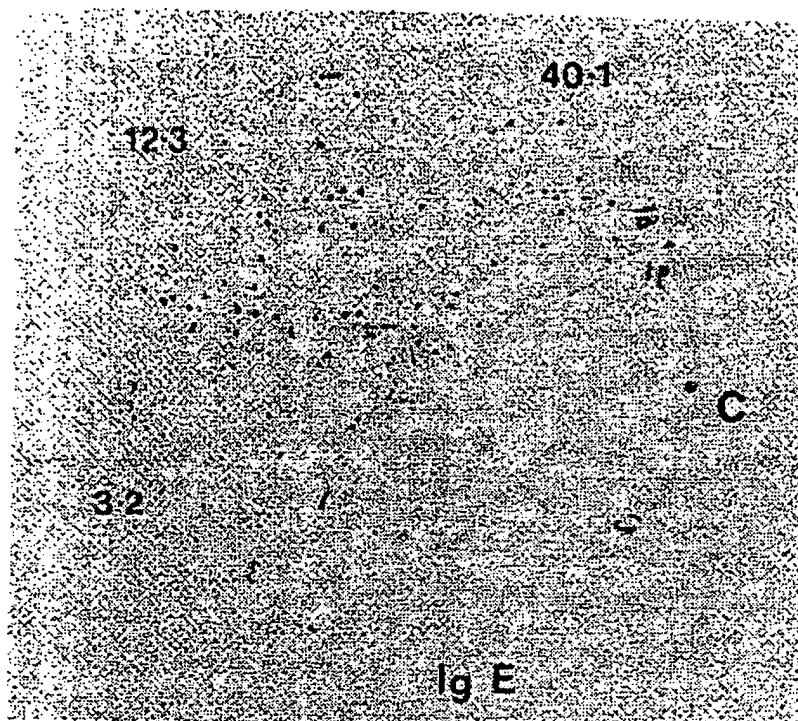
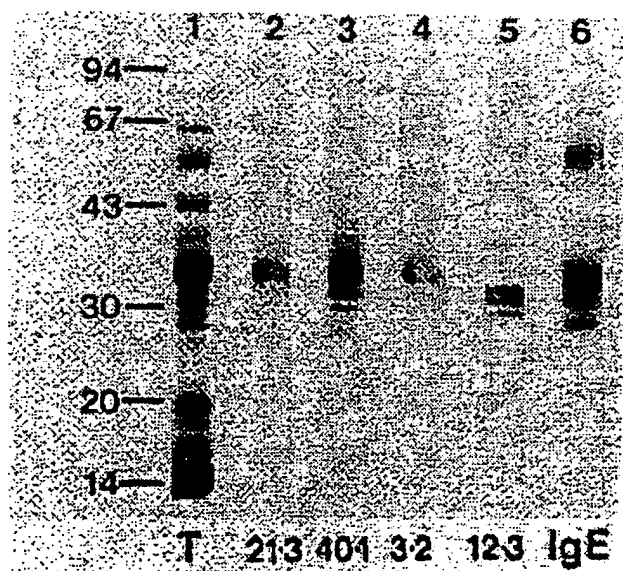
87. The antigenic fragment of claim 44 wherein said antigenic fragment does not bind immunoglobulin E specific for ryegrass pollen allergen or if binding of fragment to said immunoglobulin E occurs, such binding does not result in histamine release from mast cells of basophils.

88. A pharmaceutical composition comprising at least one antigenic fragment of purified native Lol p Ib or a derivative or homologue of said at least one antigenic fragment.

89. The isolated protein allergen or fragment thereof of claim 66 wherein said isolated protein allergen or antigenic fragment thereof is capable of stimulating T cells specific for Lol p Ib or a fragment thereof.

SUBSTITUTE SHEET

1 / 36

FIGURE 1aFIGURE 1b

SUBSTITUTE SHEET

2/36

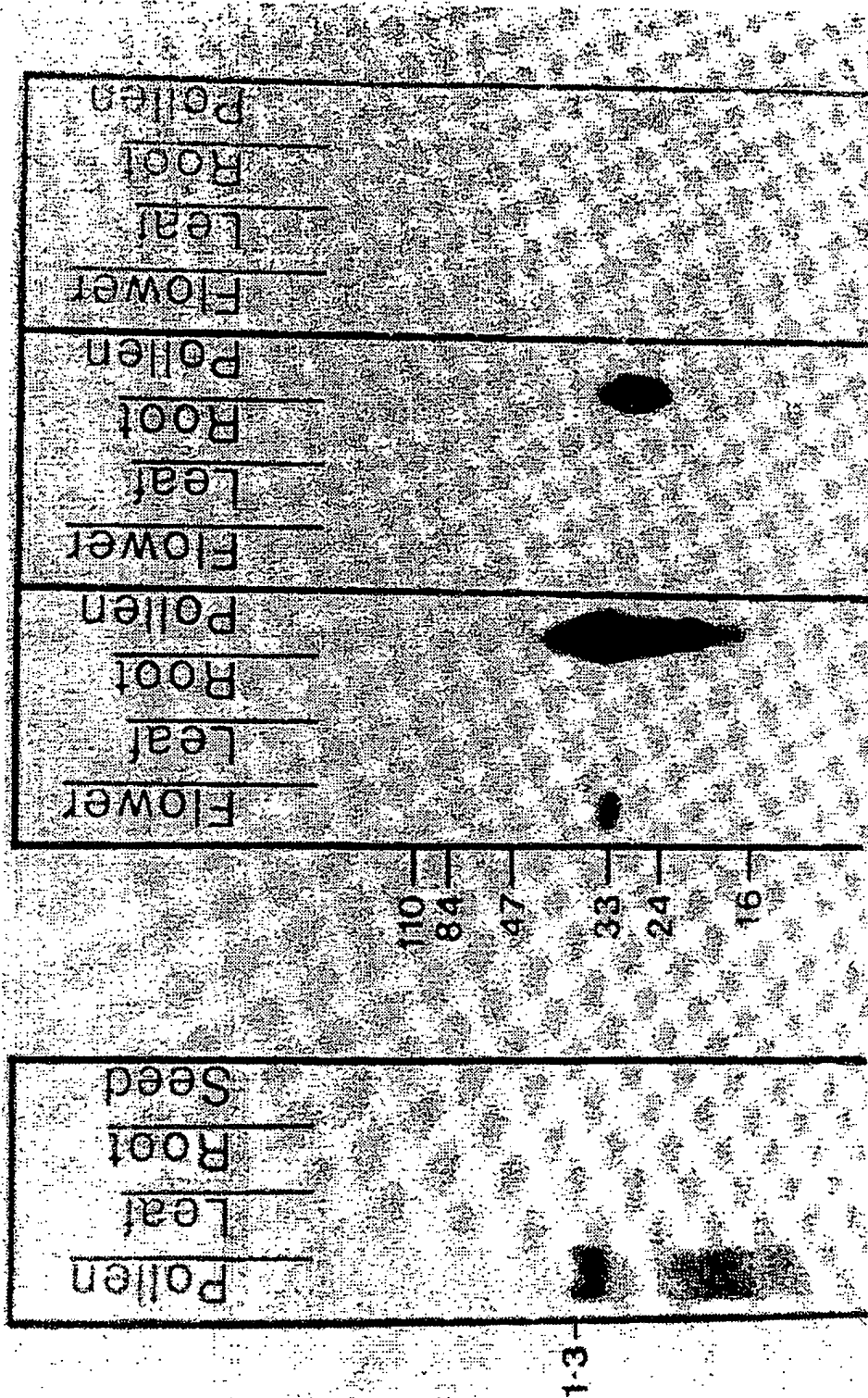


FIG 2

SUBSTITUTE SHEET

3/36

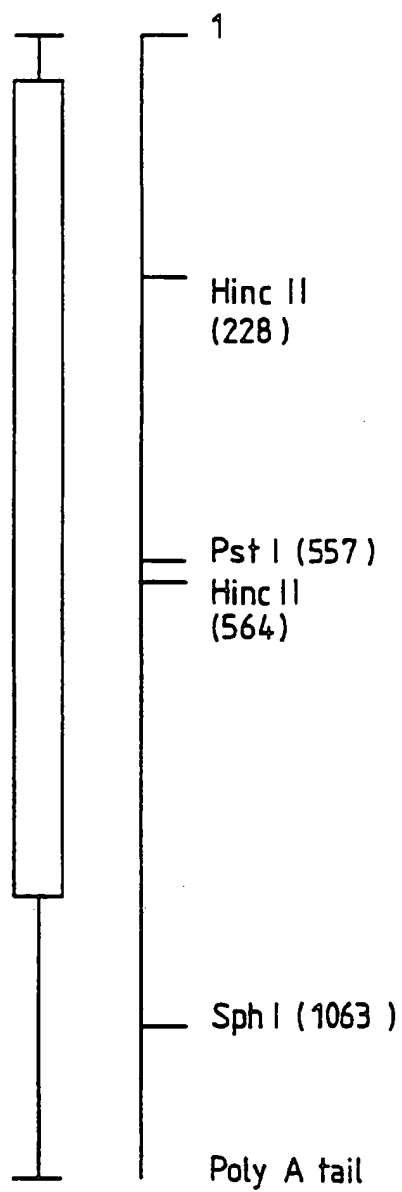
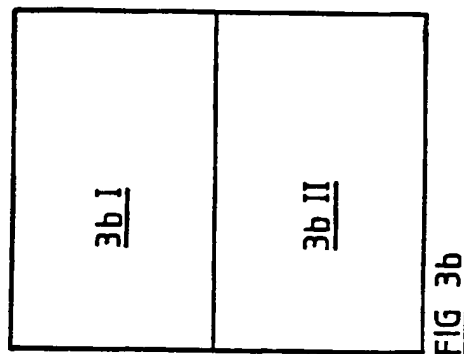


FIGURE 3a

SUBSTITUTE SHEET

4 / 36



5 / 36

Figure 3b I

CGCTATCCCT	CCCTCGTACA	AACAAACGCA	AGAGCAGCA	ATG	GCC	GTC	CAG	AAG	54
				Met	Ala	Val	Gln	Lys	
				-25					
TAC	ACG	GTG	GCT	CTA	TTC	CTC	GCC	GTG	102
Tyr	Thr	Val	Ala	Leu	Phe	Ala	Val	Ala	
-20				-15					
GCC	TCC	TAC	GCC	GCT	GAC	GCC	GCC	GCC	150
Ala	Ser	Tyr	Ala	Ala	Asp	Ala	Gly	Ala	
				1					
CCG	GCT	ACT	CCT	GCT	GCC	ACC	GCC	GCT	198
Pro	Ala	Thr	Pro	Ala	Ala	Ala	Ala	Ala	
				15					
ACC	GAC	GAG	CAG	AAG	CTG	GAC	GTC	AAC	246
Thr	Asp	Glu	Gln	Lys	Leu	Leu	Val	Asn	
					35				
GCC	GTG	GCC	GCT	GCT	GCC	CCT	CCG	GCC	294
Ala	Val	Ala	Ala	Ala	Ala	Pro	Pro	Ala	
					50				
TTC	GAG	GCC	GCT	TTC	GAG	TCC	AAG	GCC	342
Phe	Glu	Ala	Ala	Phe	Glu	Ser	Lys	Ala	

SUBSTITUTE SHEET

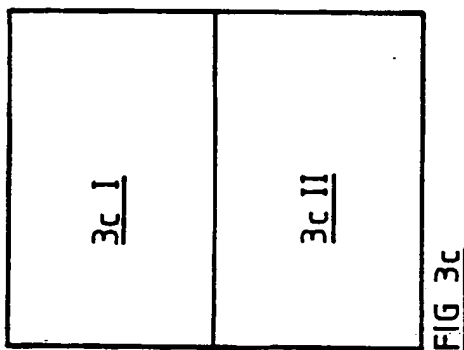
6/36

Figure 3b II

GCC Ala	GCC Ala	AAG Lys	GCA Ala	CCC Pro	GCC Gly	CTC Leu	ATC Ile	CCC Pro	AAG Lys	CTC Leu	GAC Asp	ACC Thr	GCC Ala	TAC Tyr	GAC Asp	390
GTC Val	GCC Ala	TAC Tyr	AAG Lys	GCC Ala	GCC Ala	GAG Glu	GCC Gly	GCC Ala	ACC Thr	CCC Pro	GAG Glu	GCC Ala	AAG Lys	TAC Tyr	GAC Asp	438
GCC Ala	TTC Phe	GTC Val	ACT Thr	GCC Ala	CTC Leu	ACC Thr	GAA Glu	GCG Ala	CTC Leu	CGC Arg	GTC Val	ATC Ile	GCC Ala	GCC Gly	GCC Ala	486
CTC Leu	GAG Glu	GTC Val	CAC His	GCC Ala	GTC Val	AAG Lys	CCC Pro	GCC Ala	ACC Thr	GAG Glu	GAG Glu	GTC Val	CCT Pro	GCT Ala	GCT Ala	534
AAG Lys	ATC Ile	CCC Pro	ACC Thr	GGT Gly	GAG Glu	CTG Leu	CAG Gln	ATC Ile	GTT Val	GAC Asp	AAG Lys	ATC Ile	GAT Asp	GCT Ala	GCC Ala	582
TTC Phe	AAG Lys	ATC Ile	GCA Ala	GCC Ala	ACC Thr	GCC Ala	GCC Ala	AAC Asn	GCC Ala	GCC Ala	CCC Pro	ACC Thr	AAC Asn	GAT Asp	AAG Lys	630

SUBSTITUTE SHEET

7 / 36



SUBSTITUTE SHEET

8 / 36

Figure 3c I

TTC ACC GTC TTC GAG AGT GCC TTC AAC AAG GCC CTC AAT GAG TGC ACG	678
Phe Thr Val Phe Phe Ala Phe Asn Lys Ala Leu Asn Glu Cys Thr	
175 180 185	
GGC GGC TAT GAG ACC TAC AAG TTC ATC CCC TCC GAG GCC GCG	726
Gly Gly Ala Tyr Glu Thr Tyr Lys Phe Ile Pro Ser Leu Glu Ala Ala	
190 195 200	
GTC AAG CAG GCC TAC GCC ACC GTC GCC GCC GAG GTC AAG	774
Val Lys Gln Ala Tyr Ala Ala Thr Val Ala Ala Pro Glu Val Lys	
205 210 215 220	
TAC GCC GTC TTT CAG GCC CCG CTG ACC AAG GCC ATC ACC GCC ATG ACC	822
Tyr Ala Val Phe Glu Ala Ala Leu Thr Lys Ala Ile Thr Ala Met Thr	
225 230 235	
CAG GCA CAG AAG GCC GGC AAA CCC GCT GCC GCC GCT GCC ACA GGC GCC	870
Gln Ala Gln Lys Ala Glu Lys Pro Ala Ala Ala Ala Thr Gly Ala	
240 245 250 255	
GCA ACC GTT GCC ACC GGC GGC GCA ACC GGC GCC GGT GCT GCC ACC	918
Ala Thr Val Ala Thr Glu Ala Ala Thr Ala Ala Gly Ala Ala Thr	
260 265 270	
GCC GCT GCT GGT GGC TAC AAA GCC TGATCAGCCTT GCTAATATAC TACTGAACGT	972
Ala Ala Ala Gly Tyr Lys Ala	
275	
ATGTATGTGC ATGATCCGGG CGCGGAGTGG TTTTGTTCAT AATTAATCIT CGTTTTCGTT	1032

SUBSTITUTE SHEET

9/36

Figure 3c 11

TCATGCAGCC	GGGATCGAGA	GGCCTTGCAAT	GCTTGTAATA	ATTCAATATT	TTTCATTTCT	1092
TTTTGAATCT	GTAATCCCC	ATGACAAGTA	GTGGCATCAA	GTGGCATGT	ATCACCGTTG	1152
ATGCGAGTTT	AACGATGGGG	AGTTTATCAA	AGAATTTATT	ATTAAAAAA	AAAAAAAAAA	1212
AAAAAAA	AAAAAA					1229

SUBSTITUTE SHEET

10/36

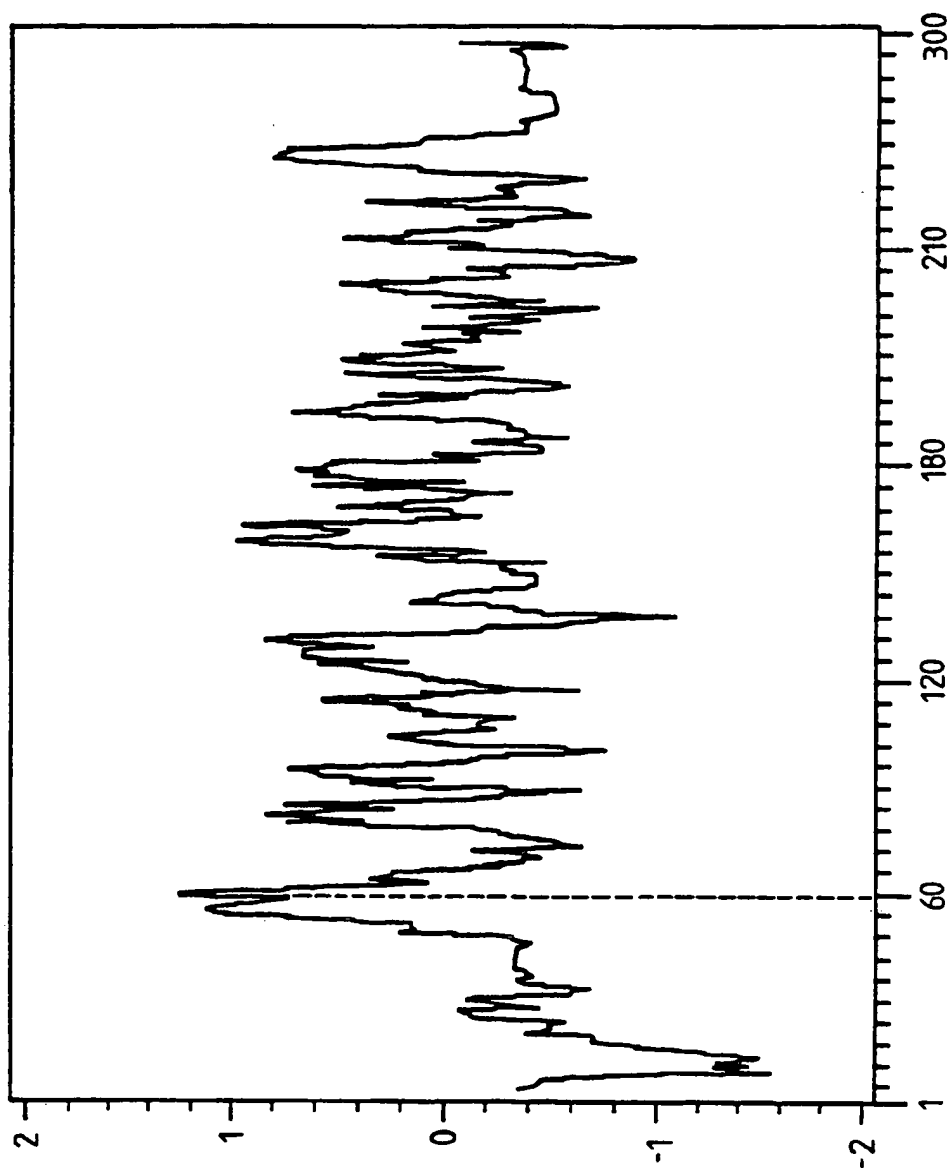
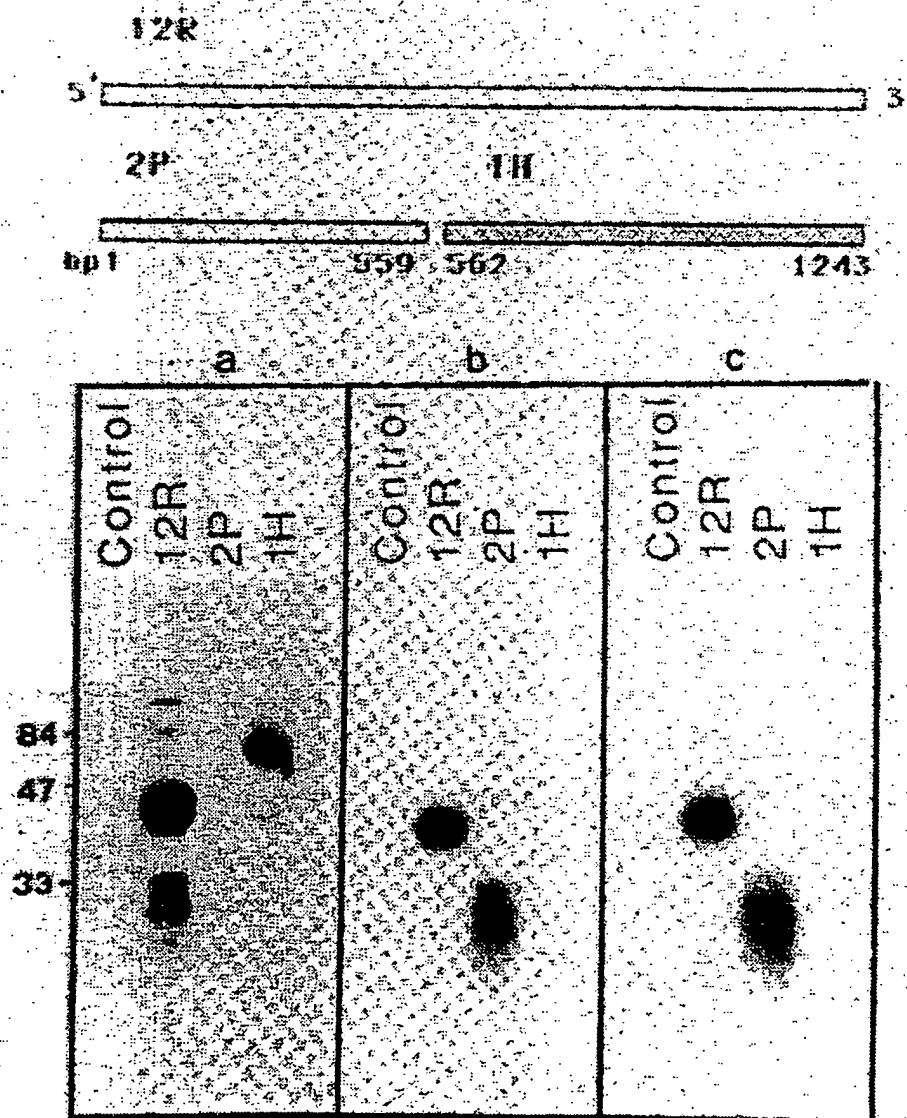


FIGURE 3d

SUBSTITUTE SHEET

11/36

FIG 4

SUBSTITUTE SHEET

12/36



FIGURE 5a



FIGURE 5b



FIGURE 5c

13/36

FIG 6

	A	B	C	D	E
1. <i>Agropyron repens</i>		—			
2. <i>Triticum aestivum</i>		—			
3. <i>Bromus inermis</i>		—			
4. <i>Agrostis alba</i>		—			
5. <i>Anthoxanthum odoratum</i>		—			
6. <i>Arrhenatherum elatius</i>		—			
7. <i>Avena sativa</i>		—			
8. <i>Holcus lanatus</i>		—			
9. <i>Phalaris arundinacea</i>		—			
10. <i>Phalaris aquatica</i>		—			
11. <i>Phleum pratense</i>		—			
12. <i>Dactylis glomerata</i>		—		—	—
13. <i>Festuca elatior</i>		—	—	—	—
14. <i>Lolium multiflorum</i>		—	—	—	—
15. <i>Lolium perenne</i>		—	—	—	—
16. <i>Poa pratensis</i>		—		—	—
17. <i>Cynodon dactylon</i>		—			
18. <i>Paspalum notatum</i>		—			
19. <i>Sorghum halepense</i>		—			
20. <i>Sorghum sudanense</i>		—			

SUBSTITUTE SHEET

14/36

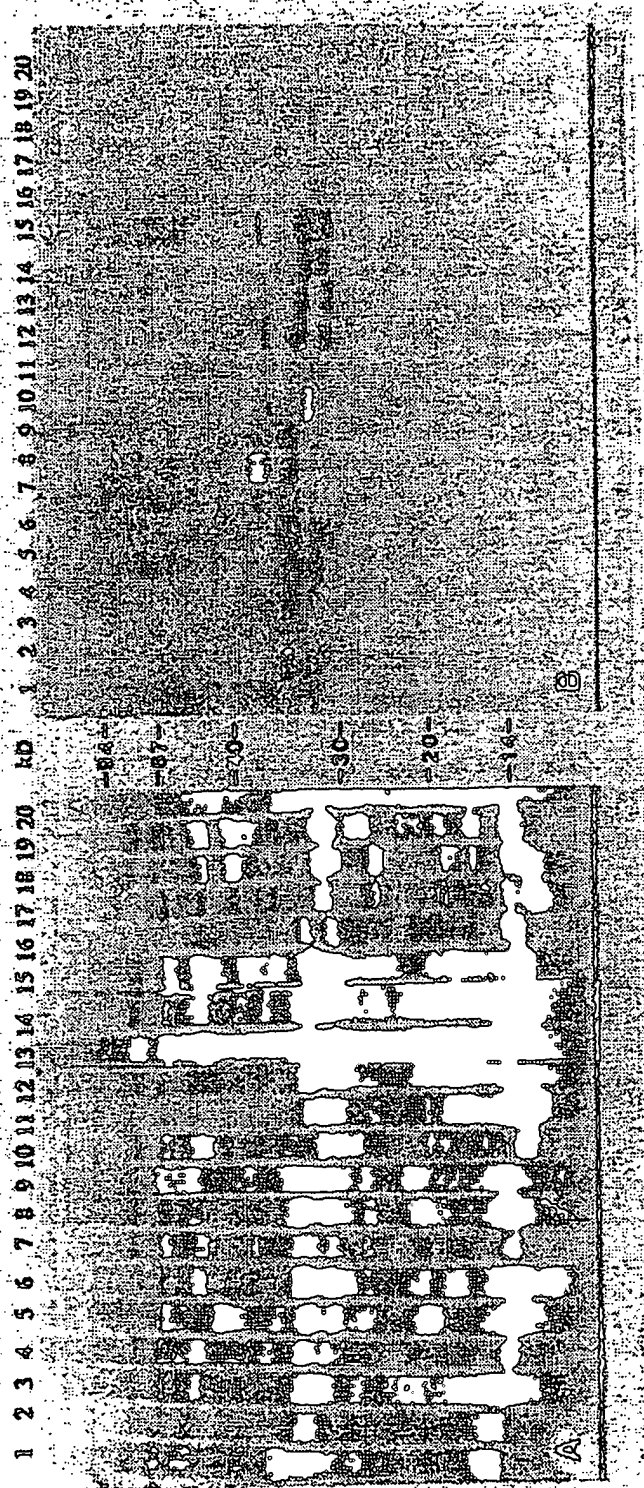


FIG 7(A,B)

15/36

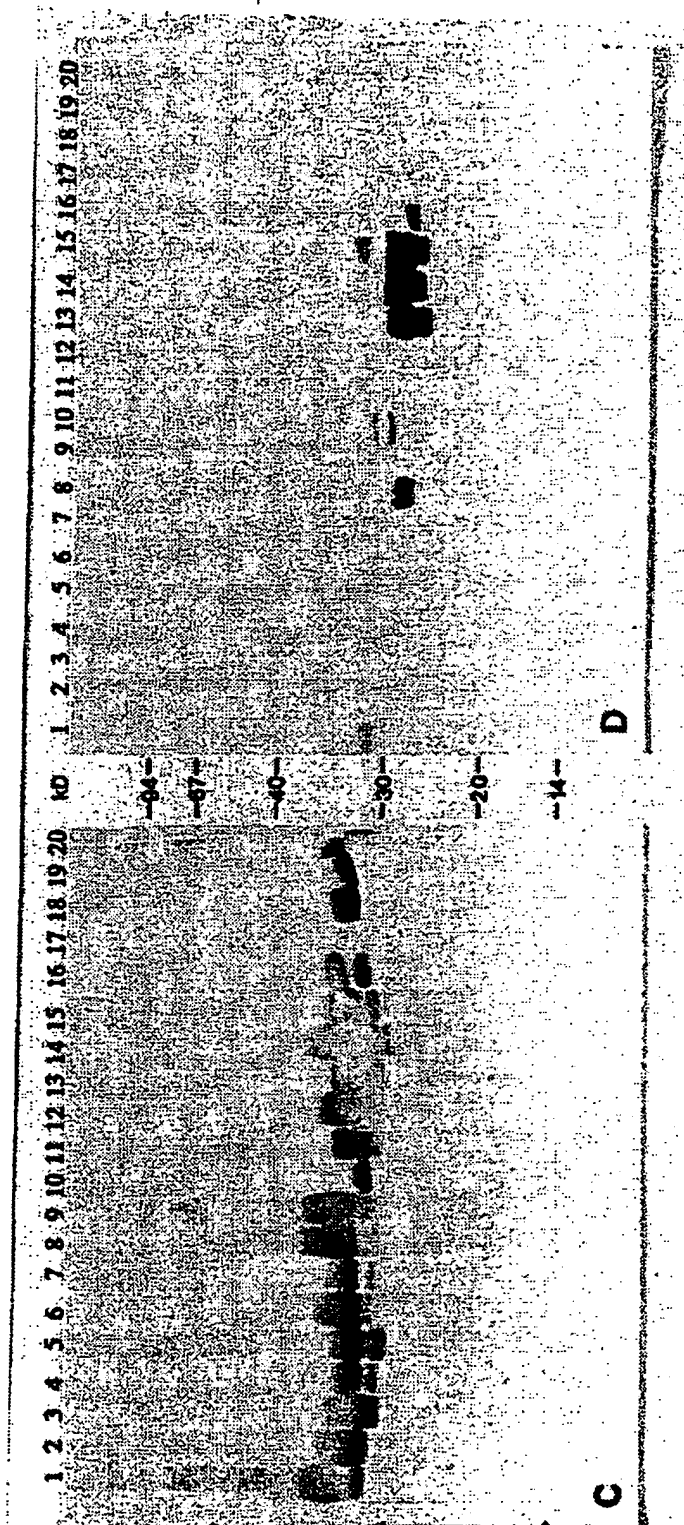


FIG 7 (C,D)

SUBSTITUTE SHEET

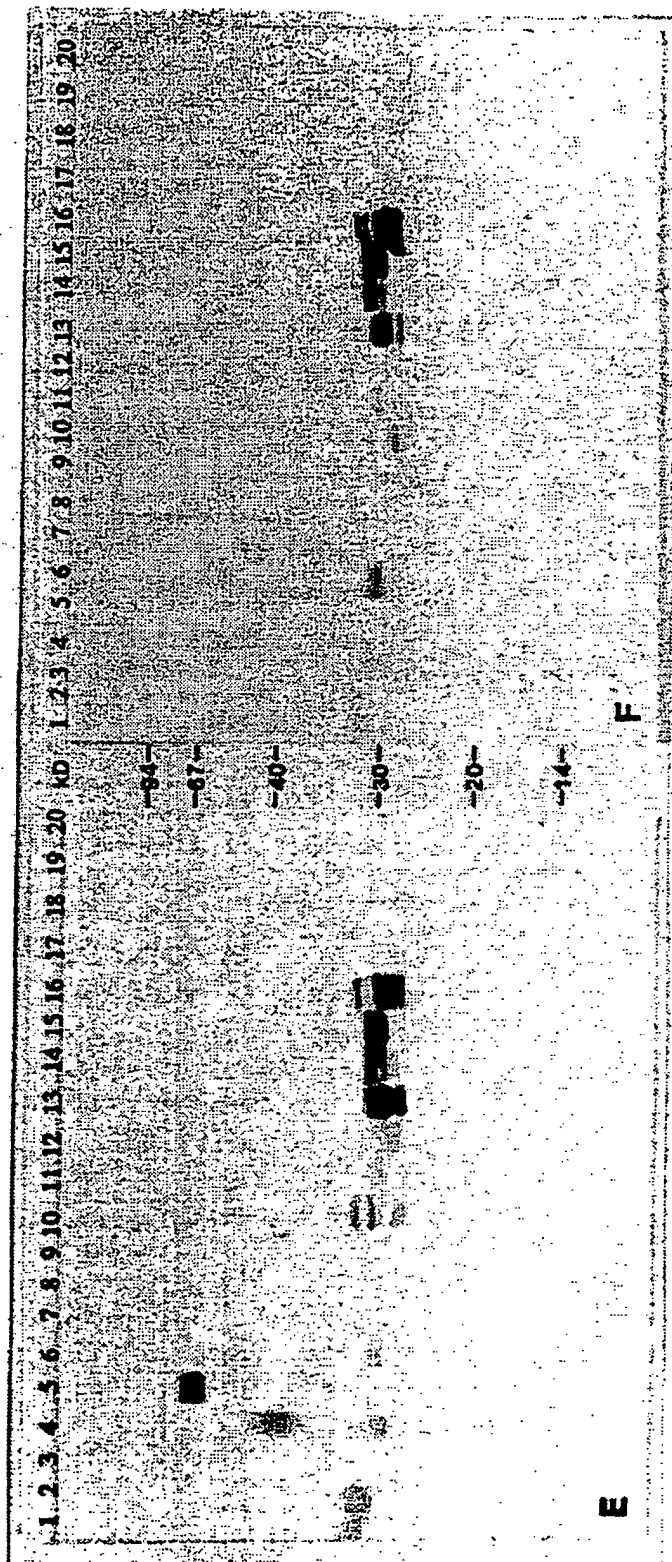


FIG 7(E,F)

17/36

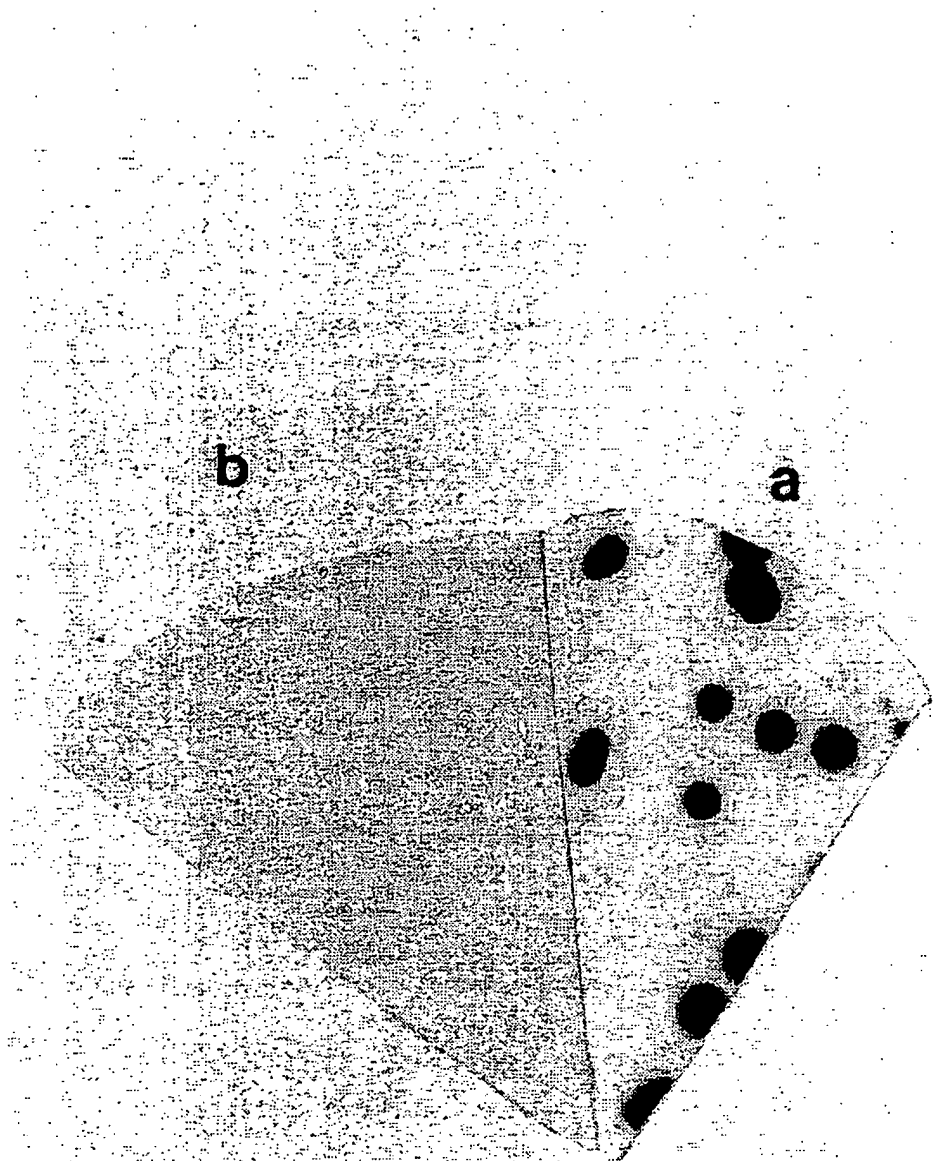


FIG 8

18/36

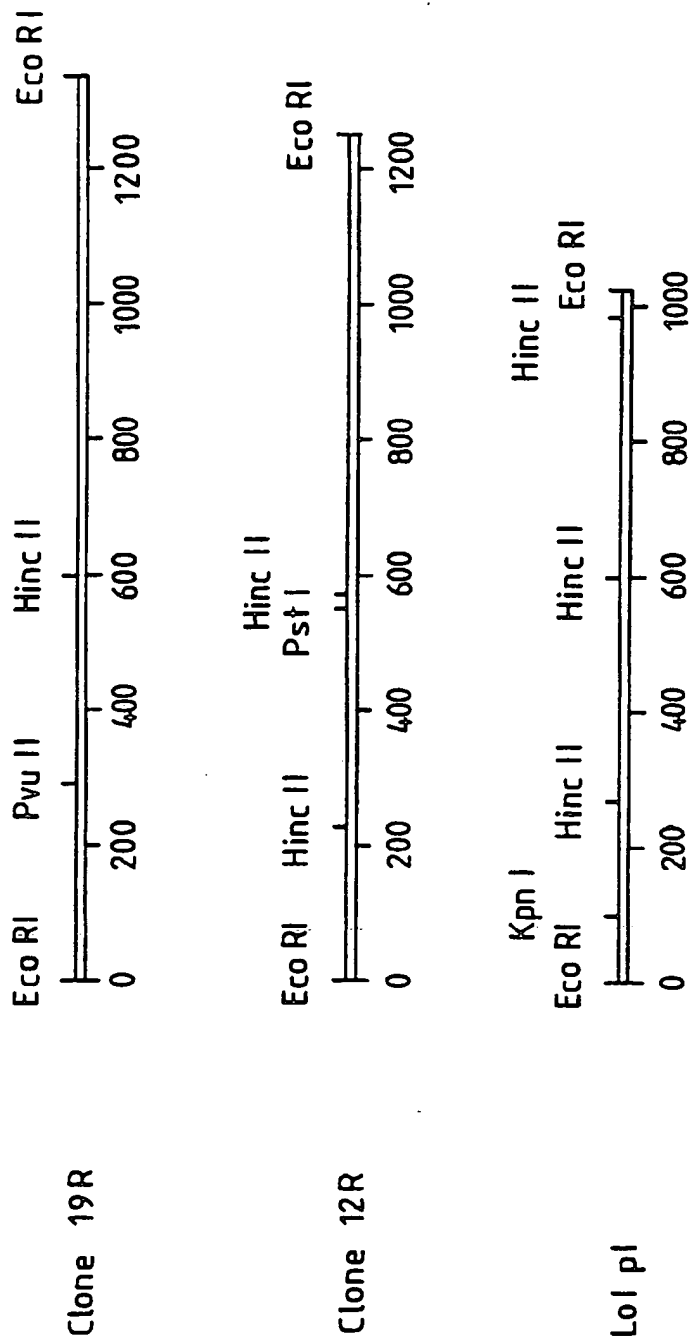
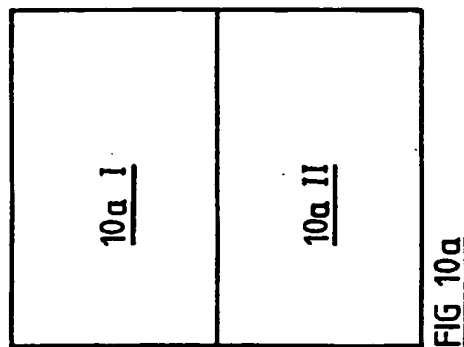


FIGURE 9

19 / 36



SUBSTITUTE SHEET

20/36

Figure 10a I

GAATTCCCCA	ACGCAAGAGC	AGCA	ATG	GCG	GTG	CAG	AAG	CAC	ACG	GTG	GCG	51
			Met	Ala	Val	Gln	Lys	His	Thr	Val	Ala	
			-25					-20				
CTT	TTC	CTC	GCC	GTG	GCC	GTG	GCG	GCC	TCC	TAC	GCT	99
Leu	Phe	Leu	Ala	Val	Ala	Gly	Pro	Ala	Ala	Ser	Tyr	
-15			-10				-5					
GCG	GAT	GCT	GCC	TAT	GCC	GCC	GCT	ACT	CCC	GCG	GCC	147
Ala	Asp	Ala	Gly	Tyr	Ala	Pro	Ala	Thr	Pro	Ala	Ala	
1			5			10				15		
GCT	ACT	GCC	GCC	ACC	ACC	GCA	ACC	CCG	GCT	ACT	CCC	195
Ala	Thr	Ala	Ala	Thr	Pro	Ala	Thr	Pro	Ala	Thr	Pro	
			20		25					30		
GCG	GTG	CCA	TCA	GGG	AAG	GCG	ACG	GAG	CAG	CTG	ATC	243
Ala	Val	Pro	Ser	Gly	Lys	Ala	Thr	Glu	Gln	Lys	Ile	
		35				40				45		
AAG	ATC	AAC	GCC	GCC	TTC	AAG	GCG	GCA	GCC	GCC	GTC	291
Lys	Ile	Asn	Ala	Gly	Phe	Lys	Ala	Val	Ala	Ala	Val	
		50				55			60			
CCA	CCA	GCT	GAC	AAG	TAC	AAG	ACG	GAA	ACC	TTC	GGC	339
Pro	Pro	Ala	Asp	Lys	Tyr	Lys	Thr	Glu	Thr	Phe	Gly	
65					70			75				
												80

SUBSTITUTE SHEET

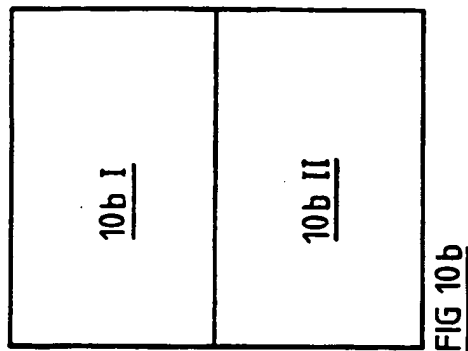
21/36

Figure 10a II

ACC AAC AAG GCC TTC GTT GAG GGC CTC GCG TCC GGC TAC GCC GAT CAA	387
Thr Asn Lys Ala Phe 86	
Val Glu Gly Leu Ala Ser 90	
Ala Asp 95	
Ala 95	
ACC AAC AAG CAG CTC ACC TCC AAG CTC GGC TTA AAG CTC GCT	435
Ser Lys Asn 100	
Leu Thr Ser Lys Leu 105	
Ala Thr 110	
Ala 110	
TAC GAG GCT GCC CAG GGC ACT CTC CCC GAG GCC AAG TAC GAT GCC TAC	483
Tyr Glu Ala 115	
Ala Thr 120	
Ala Thr 125	
Ala Lys 125	
GTC GCC ACC CTC ACC GAG GCG CTC CGC GTC ATC GCC ACC CTC GAG	531
Val Ala Thr 130	
Glu Thr 135	
Ala Thr 140	
Ala Thr 140	
GTC CAC GCC GTA AAG CCC GCG GAG GAG GTC GGC GCC ATC	579
Val His Ala Val Lys Pro 150	
Ala Thr 155	
Ala Thr 160	
Ala Thr 160	

SUBSTITUTE SHEET

22/36



SUBSTITUTE SHEET

23/36

Figure 10b I

CCC Pro	CCC Ala	CCC Ala	GAG Glu	GAG Val	GTC Gln	CTC Leu	ATC Ile	GAC Asp	AAG Lys	GTC Val	GAC Asp	GCC Ala	GCC Ala	GCG Tyr	TAC Arg	627
ACC Thr	CCC Ala	CCC Ala	ACT Thr	GCC Ala	GCC Ala	AAC Asn	GCC Ala	GCC Ala	CCC Pro	CCC Ala	AAC Asn	GAC Asp	GAC Asp	AAG Lys	TTC Phe	675
GTC Val	TTC Phe	GAG Glu	AAC Asn	ACC Thr	TTT Phe	AAC Asn	AAT Asn	GCC Ala	ATC Ile	AAG Lys	GTC Val	AGC Ser	CTG Leu	GCC Gly	GCC Ala	723
GCC Ala	TAC Tyr	ACC Ser	ACC Ser	TAC Tyr	AAG Lys	TTC Phe	ATC Ile	CCC Pro	ACC Thr	CTT Leu	GTC Val	GCC Ala	GCC Ala	GTC Gly	AAG Lys	771
CAG Gln	CCC Ala	TAC Tyr	GCC Ala	GCC Ala	AAG Lys	CAG Gln	ATC Ala	ACC Thr	GCG Ala	CCG Pro	GAG Glu	GTC Val	AAG Lys	TAC Tyr	ACT Thr	819
GTC Val	TCT Ser	GAG Glu	ACC Thr	GCG Ala	CTG Leu	AAA Lys	AAG Lys	GCC Ala	GTC Val	ACT Thr	GCA Ala	ATG Met	TCA Ser	GAG Glu	GCC Ala	867
GAG Glu	AAG Lys	GAG Glu	CCC Ala	ACC Thr	CCC Pro	CCC Ala	GCG Ala	GCT Ala	ACC Thr	ACC Thr	GCC Ala	ACC Thr	CCA Pro	ACA Thr	CCC Pro	915

SUBSTITUTE SHEET

24/36

Figure 10b II

GCG GCT GCC ACC GCC ACC GCA ACC CCC GCC GCT GCC TAC GCC ACC GCT	963
Ala Ala 278 Thr Ala Thr Ala Thr Pro Ala Ala Ala Tyr Ala Thr Ala	
ACC CCC GCC GCT GCC ACC GCA ACC CCC GCC GCT GCC ACC GCA	1011
Thr Pro 290 Ala Ala Thr Ala Thr Pro Ala Ala Thr Ala	
ACC CCC GCC GCT GCT GCC ACC GCA ACC GCA ACC GCA	1061
Thr Pro 305 Ala Ala Ala Gly Tyr Lys Val	
CATCCATCAT GCACATATAC TAACTCGTAT CTATGTGCAT GGCATGGCCG TGGGTCGAG	1121
CGATTAGCT GATAATTAT TCTTGGTTTT CGTTTCATGC ATCCGGCCGC CATCAAGCGC	1181
GTGCATGGTC AATTCGTTAT GTAATATTTG TTTTTCGATG TAAAACTAGG CCTGCGTGCC	1241
ACGCTACTCG ACTAATTAAAT GAACCGTTTT CACCTTTAAA AAAAAAGGA ATIC	1295

SUBSTITUTE SHEET

25/36

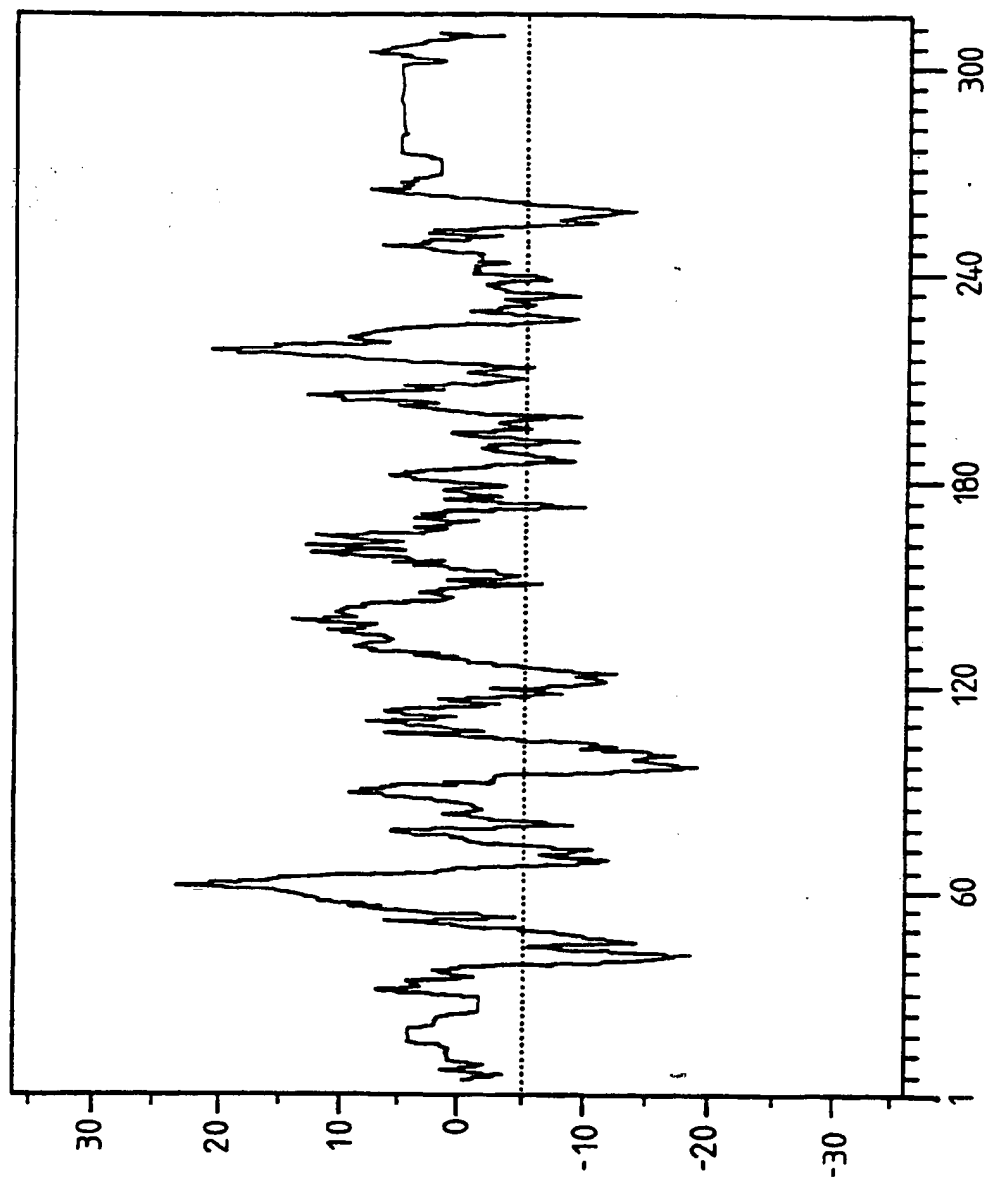
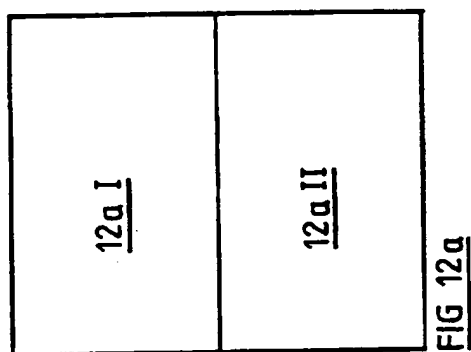


FIGURE 11

SUBSTITUTE SHEET

26 / 36



SUBSTITUTE SHEET

27/36

Figure 12a I

Clone 19R-	GAA-TT---CCCC-----AAC-----GCAAGACGACGCAATGCGCGTGCA	-38
Clone 12R-	CGCTATCCCTCCCTCGTACAAACAACGCAAGACGCAATGCGCGTGCA	-50
Clone 19R-	GAAGCACACGGTGGCGCTTTTCCTCGCCCTGGCCCTGGTGGCGGCGCCGG	-85
Clone 12R-	GAAGTACACGGTGGCTCTATTTCCTCGCCCTGGCCCTCGTGGCGGCGCCGG	-100
Clone 19R-	CCGCCTCCTACGGTGGGATGCTGGCTATGCCCGGGCCACCCCGGCTACT	-135
Clone 12R-	CCGCCTCCTACGGCGGTGACGCGGCTACACCCCGGACGCGCGGCCAC-	-149
Clone 19R-	CCCGGGCGCCCGGCTACTGCCCCCAACCCCGCCGCAACCCCGGC	-185
Clone 12R-	CCCGGCTACTCCTGCT-----GCCACCCCGGCTG---CGGC	-182
Clone 19R-	TACTCCCGCAGCGGTGCCATCAGGGAAGCGGACCGACCGAGGACGAGAAGC	-235
Clone 12R-	T-----GG-----AGGGAAGCGGACCGACCGACGACGAGAAGC	-214
Clone 19R-	TGATCCGAGAGATCAACGCCCGGCTTCAAGCGGCGCGTGGCAGCGCGCGCC	-285
Clone 12R-	TGCTGGAGGACGTCACCGCTGGCTTCAAGGACGCGTGGCGCGCGCTGCC	-264
Clone 19R-	GTCGTCCACACGCTGACAAGTACAAGACGTTTCGTGGAACCTTCGGGCAC	-335
Clone 12R-	AACGCCCTCCGGCGGACAAAGTTCAAGA---TCTTCGA-----GGC-C	-303

SUBSTITUTE SHEET

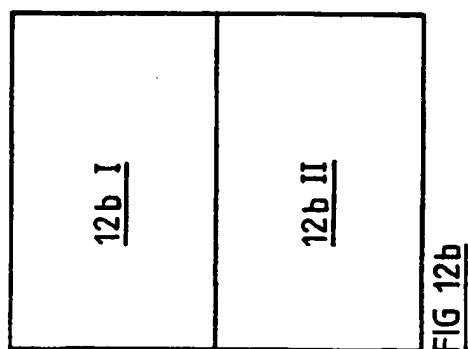
28/36

Figure 12a 11

Clone 19R-	GGCCACCAACAAGGCCTTTCGTTGAGGGCCTCGCGTCCGGCTACGCCGATC	- 385
Clone 12R-	G-CCTTCTCCGAGTCCCTCCA--AGGGCCTCCTCGCCACCTCCGGCCG-C	- 348
Clone 19R-	AAAGCAAGAACCAAGCT-CACCTCCAAGCTCGACGCCGCCCTTAAAGCTCGC	- 434
Clone 12R-	AAGGC--ACCCGGCCTCATCCCAAGCTCGACACCGCCTACGACGTCCG	- 395
Clone 19R-	TTACGAGGCTGCCCAAGGGCGCCACTCCCGAGGCCAAGTACGATGCCCTACG	- 484
Clone 12R-	CTACAAGGCCGCCGAGGGCGCCACCCCGAGGCCAAGTACGACGCCCTTCG	- 445
Clone 19R-	TCGCCACCCCTCACCGAGGGCGCTCCGGCGTCATCGCCGGCACCCCTCGAGGTC	- 534
Clone 12R-	TCACTGCCCTCACCGAAGCGCTCCGGCGTCATCGCCGGCGCCCTCGAGGTC	- 495
Clone 19R-	CACGCCGTAAAGCCCCCGCCGAGGAGGTCAAGGTCGGCGGCCA-TCCCCG	- 583
Clone 12R-	CACGCCGTCAAGCCCCCGCCACCGAGGAGGTCCCTG-CTGCTAAGATCCCCA	- 544
Clone 19R-	CCGCCGAGGTGCAGCTCATCGACAAAGTCCGACGCCCGGTACAGGACCGCC	- 633
Clone 12R-	CCGGTGAGCTGCAGATCGTTGACAAAGATCGATGCTGCCCTTCAAGATCCGA	- 594
Clone 19R-	GCCACTGCCGCCCAACGCCGCCCGCCGCAACGACAAGTTCACCGTCTTCGA	- 683
Clone 12R-	GCCACGCCGCCCAACGCCGCCCGCCGCAACGATAAGTTCACCGTCTTCGA	- 644

SUBSTITUTE SHEET

29/36

**SUBSTITUTE SHEET**

30/36

Figure 12b I

Clone 19R-	GAACACCTTTAAACAATGCCATCAAGGTGAGCCTGGCGCGCGCTACGACA	- 733
Clone 12R-	GAGTGCCTTCAACAAGGCCCTCAATGAGTGCACGGCGCGCTATCAGA	- 694
Clone 19R-	GCTACAAGTTCAATCCCAACCCCTTGTGGCCGCCGTCAAGCAGGCCCTACGCC	- 783
Clone 12R-	CCTACAAGTTCAATCCCTCCCTCGAGCGCGGTCAAGCAGGCCCTACGCC	- 744
Clone 19R-	GCCAAAGCAGGCCACCGCGCGGAGGTCAAGTACACTGTCTCTGAGACCGC	- 833
Clone 12R-	GCCACCGTCGCGCGCGCGCGGAGGTCAAGTACGCCGTCTTTGAGCGCGC	- 794
Clone 19R-	GCTGA AAAAGCGCGTCACTGCCAATGTCAGAGCGCGACAAAGGAGGCCACGC	- 883
Clone 12R-	GCTGACCAAGGCCATCAGCGCCATGACCCAGGCCACAGAGGCCGCCAAAC	- 844
Clone 19R-	CCGCC--GCGGCTGCCACCGCGCACCCCAACACCCCGCGCTGCCACCGCC	- 930
Clone 12R-	CCGCTGCCCGCGTGCCACAGCGCGCGCAAC--CGT--TGCCACCGCGC	- 888
Clone 19R-	ACCGCAACCCCGCGCTGCCCTACGCCACCGCTACCCCGCGCGCTGCCAC	- 980
Clone 12R-	GCGCAACCGCGCGCGCGG-T-----GCTGCCACCGCGCGCTGCTGG	- 929
Clone 19R-	CGCCACCGCAACCCCGCGCGTG-CCACCGCAACCCCGCGC-GCTGCTGG	- 1028
Clone 12R-	TGGCTACAAAGCCCTGATCAGCTTGCTAATA-TA-TGT	- 975

SUBSTITUTE SHEET

31/36

Figure 12b 11

Clone 19R-	TGGCTACAAAGTCTGATCAACTCTAACGGTATATATCCATCATGCCACATA	-1078
Clone 12R-	ATG-TGCATGATCCGGCGCGG--AGTGGT-TTTCGTTGATAATT-A-ATC	-1020
Clone 19R-	TACTAACTCGTATCTATGTGCATGGCATGGCCGTGGGTCAGCGATTTA	-1128
Clone 12R-	TTCCGTTTTCGTITC-AT--GCA-GCCGGGATCGAGAGGGCTTGC-ATGCT	-1065
Clone 19R-	GCTGATAATTCAATTCTTGGTT--TTCGTTTCATGCATCCGGCGCCATCA	-1176
Clone 12R-	TGTAATAATTCAATAATTTTTCATTTCTCTTT--TGAATCTGTAAATCCCCA	-1113
Clone 19R-	AGC-GCGTGCCATGGT-CAATTGTTTATGTAAATATTTGTTTTTC-GATGTA	-1223
Clone 12R-	TGACAACTAGTGGGATCAAGTCGGCATGTATCACC-GTTGATGCCA-GTT	-1161
Clone 19R-	AACTAG-GCCTGCGTGCCACGCTACTCGACTAATTAAATGAACCGTTTTC	-1272
Clone 12R-	TAAAGATCGCGACTTTATCAAGAATT-ATTA-TTAAAAAATAAAAAAA	-1209
Clone 19R-	ACCTTTAAAAAATAACGAATTC	-1295
Clone 12R-	AAA---AAAAAATAAAAAAA	-1229

SUBSTITUTE SHEET

32/36

Figure 13

Clone 19R-	MAVQKHTVALFLAVALVAGPAAASYAADAGYAPATPATPAAPATAATPATP	-50
Clone 12R-	MAVQKHTVALFLAVALVAGPAAASYAADAGY-----TPAAAATP	-38
Clone 19R-	ATPATPAAVPSGKATTEECKLIEKINAGFKA AVAAAVVPPADKYKTEVE	-100
Clone 12R-	ATPATPAAGGKATTDEQKLLLEDV NAGFKA AVAAANAPPADKFKIFEA	-88
Clone 19R-	TFG-----TATNKAFVEGLASGYADQSKNQLTSKLDAAALKLAYEAAQ	-162
Clone 12R-	AFSESSKGLLATSAAKAPG-----LIPKLD TAYDVAYKAAE	-124
Clone 19R-	GATPEAKYDAYVATL TEALRVIA GTLEVHAVKPAEEVKVGAIPAAEVQL	-192
Clone 12R-	GATPEAKYDAFVTAL TEALRVIA GALEVHAVKPA TE EVPAAKIPIGELQI	-174
Clone 19R-	IDKVDAAYRTAATAANAAPANDKFTTVFENTFNNAIKVSLGAAYDSYKFIB	-242
Clone 12R-	VDKIDAAF KIAATAANAAPTNDKFTTVFESAFNKALNECTGAYETYKFIP	-224
Clone 19R-	TLVAAVKQAYAAKQATAPEVKYTVSETALKKAVTAMSEASKEATPAAAAT	-292
Clone 12R-	SLEAAVKQAYAAATVAAAPEVKYAVFEAALT KAITAMTQAQKAGKPAAAA-	-233
Clone 19R-	ATPTPAAATATATPAAAYATATPAAATATATATPAAATATPAAAGCYKV	-339
Clone 12R-	ATGAATVATGAATAAAGAAT-AAAGGYKA	-301

SUBSTITUTE SHEET

33/36

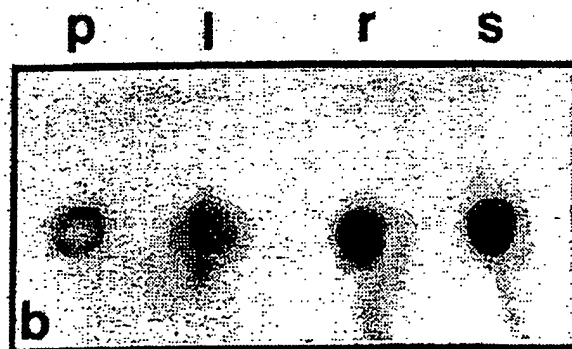
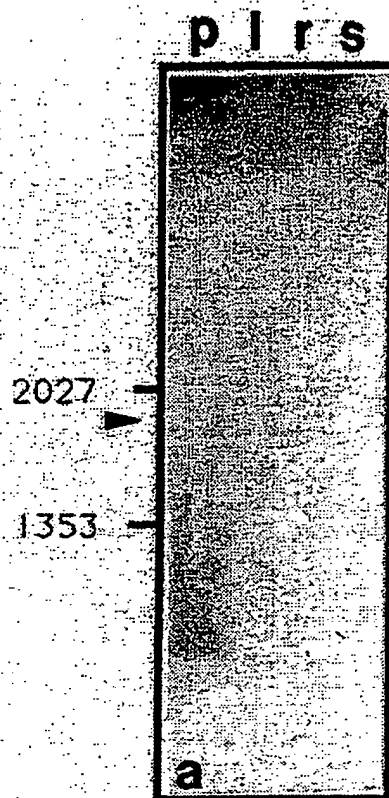


FIG 14

SUBSTITUTE SHEET

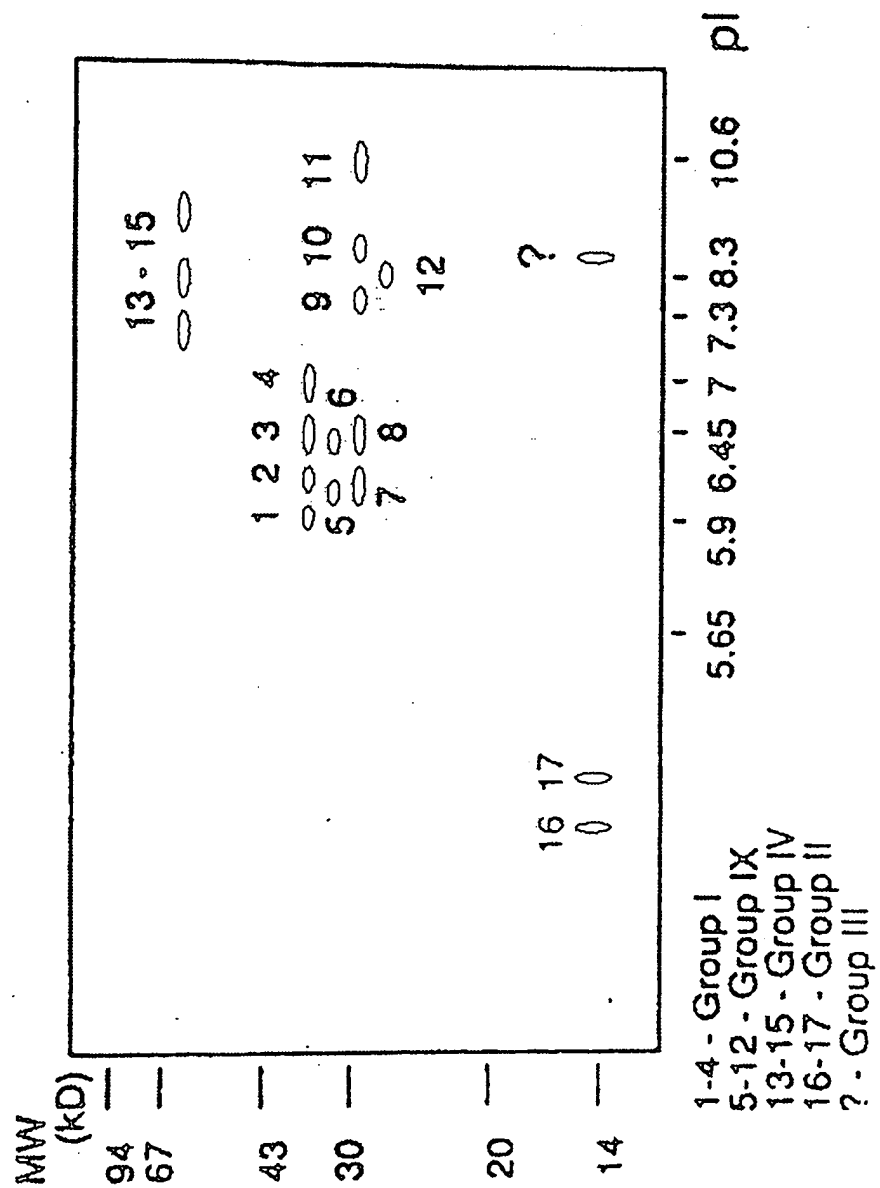
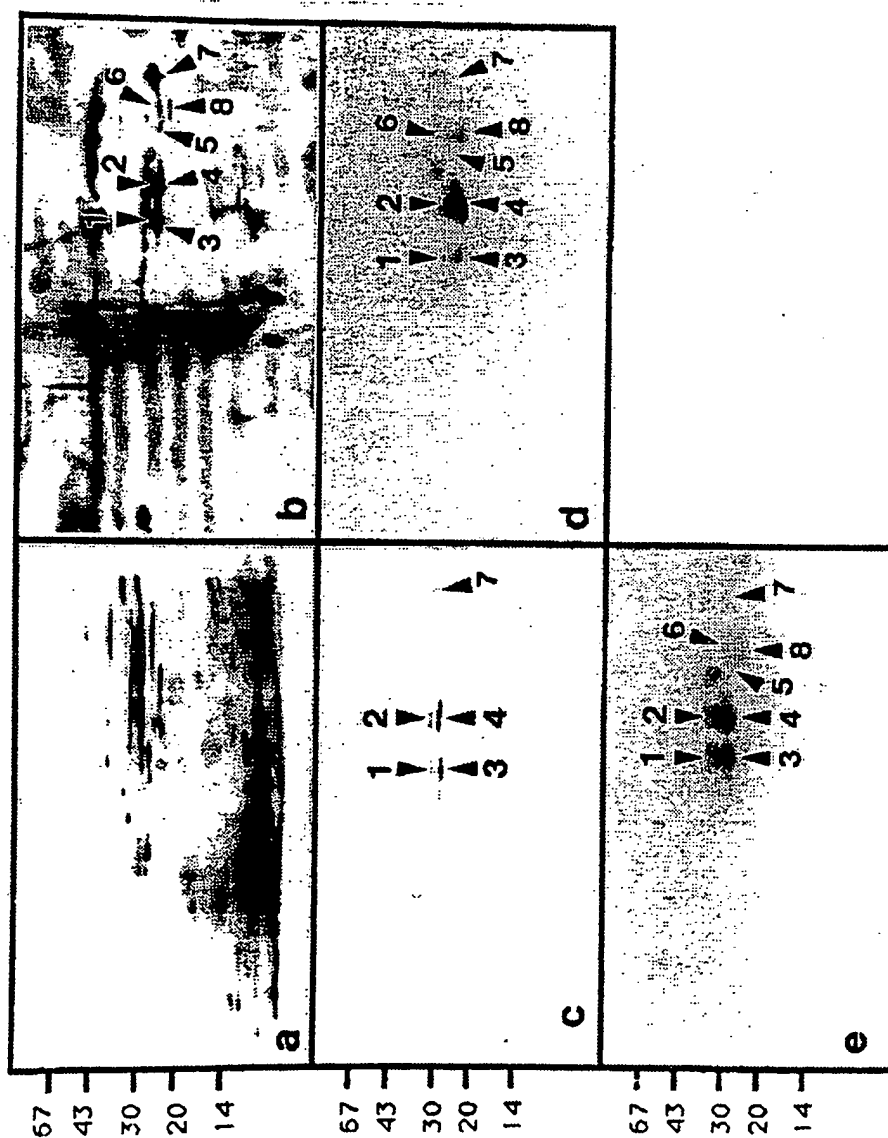


FIG 15

SUBSTITUTE SHEET

35/36

FIG 16




SUBSTITUTE SHEET

INTERNATIONAL SEARCH REPORT

International application No.

PCT/AU92/00430

A. CLASSIFICATION OF SUBJECT MATTER Int. Cl. ⁵ C12N 15/29; C12P 21/08; A61K 39/36 According to International Patent Classification (IPC) or to both national classification and IPC				
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) IPC DERWENT ONLINE WPAT, CASA, C12N, KEYWORDS: ALLERGEN, ANTIGEN, RYEGRASS, RYE (W)GRASS, LOLIUM, L(W)PERENE. Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched AU C12N 15/29, C12P 21/08, A61K 39/36 Electronic data base consulted during the international search (name of data base, and where practicable, search terms used) DERWENT ONLINE, WPAT, CASA, BIOT; KEYWORDS:- ALLERGEN, ANTIGEN, RYEGRASS, RYE(W)GRASS, LOLIUM, L(W)PERENE.				
C. DOCUMENTS CONSIDERED TO BE RELEVANT				
Category ^o	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.		
X	AU-A-84083/91 IPC C12N 15/29, THE UNIVERSITY OF MELBOURNE, 5 MARCH 1992 (05.03.92)	1-89		
A	AU-A-31644/89 IPC C07K 13/00, THE UNIVERSITY OF MELBOURNE, 28 SEPTEMBER 1989 (28.09.89)	1-89		
X	BIOCHEM J VOL 234, 1986, PP 305-310, COTTAM G.P. et al, PHYSICOCHEMICAL AND IMMUNOCHEMICAL CHARACTERISATION OF ALLERGENIC PROTEINS FROM RYEGRASS (<u>LOLIUM PERENNE</u>) POLLEN PREPARED BY A RAPID AND EFFICIENT PURIFICATION METHOD. (continued)	1-89		
<div style="display: flex; justify-content: space-between; align-items: center;"> <div style="display: flex; align-items: center;"> <input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C. </div> <div style="display: flex; align-items: center;"> <input type="checkbox"/> See patent family annex. </div> </div>				
<table style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 50%; vertical-align: top;"> <p>* Special categories of cited documents :</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </td> <td style="width: 50%; vertical-align: top;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"&" document member of the same patent family</p> </td> </tr> </table>			<p>* Special categories of cited documents :</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p>	<p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"&" document member of the same patent family</p>
<p>* Special categories of cited documents :</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p>	<p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"&" document member of the same patent family</p>			
Date of the actual completion of the international search 19 November 1992 (19.11.92)		Date of mailing of the international search report 3: Nov 1992 (20.11.92)		
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200 WODEN ACT 2606 AUSTRALIA Facsimile No. 06 2853929		Authorized officer  BERNARD NUTT Telephone No. (06) 2832364		

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category [*]	Citation of document, with indication, where appropriate of the relevant passages	Relevant to Claim No.
X	Journal of Allergy and Clinical Immunology, VOL 78, No 6, 1986, PP 1190-1201, L.R. Friedhoff et al. "A study of the human immuno response to <u>Lolium perenne</u> (Rye) pollen and its components, Lol p1 and Lol p11 (Rye I and Rye II)".	1-66
X	Molecular Immunology, VOL 23, No 12, 1986, PP 1281-1288. KAHN C.R. & MARSH D.G. "Monoclonal antibodies to the major <u>Lolium perenne</u> (Rye Grass) pollen allergen Lol pI (Rye I)	1, 23, 25 84-86
X	Int. Arch. Allergy, Appl. Immun. VOL 78, 1985 pp 300-304, Singh M.B and Knox R.B, "Grass pollen allergens: antigenic relationships detected using monoclonal antibodies and dot blotting immunoassay".	1, 23, 25 84-86

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/AU92/00430

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report						Patent Family Member					
AU	84083/91		WO	9203550							
AU	31644/89		DK	2281/90		EP	406286		WO	8909260	
END OF ANNEX											

This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☒ FADED TEXT OR DRAWING
- ☒ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☐ SKEWED/SLANTED IMAGES
- ☐ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☐ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☒ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.